THE FREQUENCY OF PRE-CORE GENE MUTATIONS IN CHRONIC HEPATITIS B INFECTION : A STUDY OF MALAYSIAN SUBJECTS

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Abstract. A retrospective study was carried out to determine the frequency of the pre-core stop codon mutant virus in a group of chronic hepatitis B carriers: 81 cases were considered [33 hepatits B e antigen (HBe) positive and 48 HBe negative]. All of the HBe positive cases had detectable viral DNA by hybridization analysis; in the case of the HBe negative cases, one third had detectable viral DNA by hybridization analysis and two thirds had HBV DNA detectable by polymerase chain reaction (PCR) amplification. Pre-core stop codon mutant detection was carried out on all specimens using allele-specific oligonucleotide hybridization following PCR amplification of the target sequence. The pre-core mutant was detected in 13/33 (39.4%) of HBe positive cases and in 32/48 (66.7%) of HBe negative cases. Sequence analysis was carried out on 8 of the 16 HBe negative specimens that did not carry the pre-core mutant virus to determine the molecular basis for the HBe minus phenotype in these cases: the 1762/1764 TA paired mutation in the second AT rich region of the core promoter was detected in five cases; a start codon mutation was detected in one case. The predominant mutation resulting in the HBe minus phenotype in our isolates was the 1896A pre-core ("pre-core stop codon") mutation; other mutations responsible for the phenotype included the core promoter paired mutation and pre-core start codon mutation. In view of the high frequency of the pre-core mutant virus, sequence analysis was performed to determine the virus genotype on the basis of the nucleotide sequence of codon 15. The sequences of 21 wild type virus (14 HBe positive and 7 HBe negative cases) were examined: 15 were found to be codon 15 CCT variants (71.4%); the frequency in the HBe positive group was 12/14 (85.7%), while that in the HBe negative group was 3/7 (42.9%). The high frequency of the codon 15 CCT variant in association with the frequent occurrence of the pre-core mutant in our isolates concurs with the results of other studies.

INTRODUCTION

Hepatitis B infection is one of the most common infectious diseases in the world and is relatively highly prevalent in many parts of Asia and Africa. The infection is endemic in Malaysia and throughout Southeast Asia. Infection with the hepatitis B virus (HBV) is characterized by a variable tendency towards viral persistence in the host; serious consequences of their viral persistence include the development of chronic liver disease and progression to liver cell carcinoma. Another characteristic feature of HBV is its propensity for the acquisition of mutations in different regions of its genome; these regions include pre-core/core, surface, polymerase, and X genes. Interest in the mutations present in the pre-core region of the virus has been stimulated by the association of these mutations with the development of the hepatitis B e (HBe) minus phenotype: this association is caused by the introduction of a stop codon in the pre-core region, which results in the loss of expression of the HBe antigen (Brunetto *et al*, 1989; Carman *et al*, 1989; Akahane *et al*, 1990; Tong *et al*, 1990; Ulrich *et al*, 1990; Bonino *et al*, 1991). This mutation is reported mainly from among patients of the Mediterranean and Asian regions (Akahane *et al*, 1990; Carman *et al*,

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1992; Chan *et al*, 1999; 2000; Chu *et al*, 1996; Okamoto *et al*, 1990; Santantonio *et al*, 1991). The clinical and pathogenic significance of the pre-core mutant virus relative to the wild type (WT) virus have been the subject of much investigation (Brunetto *et al*, 1991; 1993; Omata *et al*, 1991; Wands *et al*, 1991; Brown *et al*, 1992; Naoumov *et al*, 1992; Zhang *et al*, 1996; Inoue *et al*, 1998).

It is widely believed that the pre-core mutant virus emerges as a result of immune selection during the course of infections. The accumulation of greater numbers of mutant strains than WT virus in chronic HBV infection implies that the mutant strains have a comparative survival advantage. In the case of the pre-core mutant, it is suspected that switching off the translation of the HBe antigen allows the virus to evade immune pressure; furthermore, the mutation may result in the increased efficiency of replication of the virus. The pre-core region consists of 87 nucleotides (29 codons) that precede, and that are in phase with, the core gene. The pre-core region contains within its sequence the epsilon encapsidation signal (∈) that spans nucleotides 1852-1930; the ∈ sequence consists of a number of nested inverted repeats which can base-pair to form a stem loop structure comprising an upper and a lower stem (Junker-Niepmann *et al*, 1990; Pollack and Ganem, 1993; Wang and Seeger, 1993). The lower stem of this putative structure includes both the 1858-1896 (T-G) base pair and the 1855-1899 (T-G) base pair. An 1896G→A point mutation results in the replacement of the less stable 1858-1896 (T-G) pair by the more stable T-A pair, thereby conferring improved replication efficiency to the mutant virus (Tong *et al*, 1992; 1993; Laskus *et al*, 1994; Lok *et al*, 1994); this explains the infrequent occurrence of the pre-core mutation in geographical regions where the predominant virus genotype carries a C at 1858 (genotypes A and F) instead of a T (genotypes B, C, D, E) (Brunetto *et al*, 1993; Li *et al*, 1993; Rodriguez-Frias *et al*, 1995; Lindh *et al*, 1997) an 1896G→A point mutation in this case will render the mutant virus incompatible for pairing with the C at nucleotide 1858.

Data related to the pre-core mutant virus have been derived mostly from studies in Mediterranean (Brunetto *et al*, 1989; Carman *et al*, 1989; Santantonio *et al*, 1991) and Asian countries (Akahane *et al*, 1990; Carman *et al*, 1992; Okamoto *et al*, 1990; Chu *et al*, 1996; Chan *et al*, 1999; 2000). The objective of this study was an evaluation of the frequency of the pre-core gene mutation that results in the formation of a stop signal at codon 29 in chronic hepatitis B carriers in Malaysia. The distribution of the hepatitis B virus genotype based on the nucleotide (nt) sequence of codon 15 (nt1856 - 1858) of the viral genome was assessed to determine the association between the frequency of pre-core mutations and the virus genotype. The results of a limited study of the type and distribution of mutations responsible for the HBe minus phenotype in our patients are also presented.

MATERIALS AND METHODS

Serum samples

The sera used for the study were taken from chronic hepatitis B carriers who had been routinely followed-up in our gastroenterology clinic; the specimens were retrieved from archives. Ninety-three serum specimens were studied: 33 from known hepatitis B e antigen (HBe) positive cases (selected randomly) and 60 from known HBe negative subjects. All the HBe positive cases had detectable levels of HBV DNA: a mean of 265 pg/200 μl of serum (range: 2 to >1,000 pg/200 μl of serum). The HBe negative cases were selected to include a number of specimens (n=33) that tested positive for HBV DNA (mean~60 pg/200 μl; range: 1-850 pg/200 μl) by dot blot hybridization (DBH) analysis (Yap *et al*, 1994). Of the remaining 27 HBe negative sera (*ie* samples negative for HBV DNA by DBH), 18 were positive for HBV DNA following *in vitro* amplification by the polymerase chain reaction (PCR) coupled with detection using agarose gel electrophoresis and ethidium bromide stain-

ing (AGE-EtBr). A total of 51 HBe negative cases tested positive for the HBV virus genome; the 9 cases that did not have detectable virus by PCR were excluded from further analysis.

Most of the subjects included in this study were ethnic Chinese (N = 75). One possible reason for the disproportionate number of Chinese subjects in our study group is the higher frequency of chronic hepatitis B in ethnic Chinese compared with the other two major ethnic groups (Malays and Indians) in the local population (unpublished data). Of the HBe negative subjects, 42 of 51 cases had seroconverted by the time of their recruitment to our follow-up clinic; the remaining 9 cases seroconverted during follow-up. At the time of testing for the pre-core mutation, the average duration of seroconversion in this group was 14.7 months (range: 6 to 43 months).

Pre-core mutation testing

Specimens were screened for pre-core mutations using allele-specific oligonucleotide (ASO) hybridization based on the protocol described by Li *et al* (1990). Briefly, 100 μl of serum were subjected to proteinase K and Tween 20 treatment followed by extraction using phenol/chloroform. The extracted DNA was ethanol precipitated and stored at -20ºC until analysis. The viral DNA was amplified using the polymerase chain reaction (PCR) and primers (M3 and 3C) derived from the pre-core/core regions and spanning nucleotides 1730 and 2458 (Carman *et al*, 1989). The nucleotide sequences of the primers are given in Fig 1. Following amplification, the product was examined by agarose gel electrophoresis before hybridization analysis using oligonucleotide probes specific for the wild type (WT) and mutant (MT) viruses. Probes were end-labeled with γ-^{32}P-ATP using T4 polynucleotide kinase (Clontech Laboratories Inc). The probe sequences for the WT (M0) and the two mutant virus strains (M1 and M2) are given in Fig 1. *In vitro* amplification was carried out for 30 cycles under the following conditions: denaturation at 94ºC for 1 minute, primer annealing at 55ºC for 1 minute; extension at 72ºC for 2 minutes. Hybridization of the labeled probe to target sequence was performed for 2 hours at 60ºC, using a hybridization solution containing 6xSSC, 5x Denhardt's solution, 0.5% SDS, and 0.5 mg/ml salmon sperm DNA. Following hybridization, a high stringency wash was given using 2xSSC, 0.1% SDS for 10 minutes, 1xSSC, 0.1% SDS for 10 minutes and 0.2xSSC, 0.1% SDS for another 10 minutes sequentially; the wash temperature was 60ºC for the M0 and M1 probes and 55ºC for the M2 probe. Detection of hybridization was achieved by autoradiography. The hybridization analysis using the allele-specific probes distinguishes the wild type virus from mutant viruses that carry an 1896G→A mutation (M1 mutant) or an 1899G→A mutation as well as from that carry the 1896G→A mutation (M2 mutant). Both mutant strains result in the HBe minus phenotypes.

Nucleotide sequences of Primers M3, 3C and BC1

M3	5′CTGGGAGGAGTTGGGGGAGGAGATT	(1730 - 1754)
3C	5′CTAACATTGAGATTCCCGAGA	(2458 - 2439)
BC1	5′GGAAAGAAGTCAGAGGCAA	(1974 - 1955)

Nucleotide sequence of the mutation detection ologonucleotides (probe sequences)

TGGGTGGCTTTGGGGCATGGAC	(1887 - 1908)	Wild type (M0)
GGGTGGCTTTAGGGCATGGAC	(1888 - 1908)	Mutant (M1)
GGGTGGCTTTAGGACATGGAC	(1887 - 1908)	Mutant (M2)

Fig 1–Nucleotide sequences of the mutation detection oligonucleotides and primers used in PCR amplification and sequence analysis.

Confirmatory DNA sequencing

Specificity of hybridization was confirmed by direct sequence analysis of PCR products in 30 samples (21 containing only WT virus; 6 containing both WT and mutant viruses; and 3 containing only mutant virus). The pre-core/core region spanning nucleotides 1730 and 1974 were sequenced following PCR amplification of the target sequence (Nt1730 - 2458).

The sequence of the sequencing primer, BC1, is given in Fig 1. Sequencing was carried out using the ABI Prism BigDye terminator cycle sequencing kit (PE Applied Biosystems). The concordance of results between the hybridization analysis and the sequencing was 93.3% (28 of 30 samples); the two samples with discordant results were positive for both the WT and the pre-core mutant based on the hybridization analysis; on sequencing, only the WT virus sequence was found. Examination of the blots showed that the predominant virus was the WT strain, explaining the failure of detection of the mutant virus on sequencing.

RESULTS

Specific hybridization for the wild type (WT) and/or mutant (MT) viruses was achieved for all 33 HBe positive sera. In the case of the HBe negative sera, specific hybridization was successful in 32 of 33 sera that were HBV DNA positive by DBH analysis, and in 16 of 18 sera that were HBV DNA positive by PCR/AGE-EtBr analysis. Therefore, only 48 of 51 HBe negative samples with detectable HBV DNA yielded results following the pre-core mutation screen; 3 samples failed to hybridize with the oligoprobes in the mutant screen assay. The basis for the lack of hybridization was demonstrated in one sample for which the sequence data was available: the hepatitis virus in this case was a WT variant with CCC at codon 15; a G→A mutation at nt1898, accompanied by a compensatory C→T change at nt1856, explained the failure of hybridization with any of the specific probes.

The results of the pre-core mutation screen are shown in Table 1. The pre-core mutation was detectable in 13 of 33 HBe positive samples (39.4%) compared with 32 of 48 HBe negative samples (66.7%). All HBe positive specimens

Table 1
Distribution of wild type and pre-core mutant hepatitis B virus on the basis of the HBe serological status.

Genotype	Hepatitis B e status		Total
	HBeAg positive (%)	HBeAg negative (%)	
Wild type	20 (60.6)	16 (33.3)	36
Mixed	13 (39.4)	21 (43.8)	34
Mutant	0	11 (22.9)	11
Total	33	48	81

Table 2
Distribution of wild type and pre-core mutant hepatitis B virus in HBe negative cases on the basis of viral load.

Genotype	HBe Ag negative cases with HBV DNA detectable by		Total
	Hybridization analysis (%)	*In vitro* amplification (%)	
Wild type	5 (31.3)	11 (34.4)	16
Mixed	9 (56.3)	12 (37.5)	21
Precore mutant	2 (12.5)	9 (28.1)	11
Total	16	32	48

Table 3
Evolution of the wild type and pre-core mutant hepatitis B virus with progression from HBeAg positive through the HBe window to the anti-HBe positive state.

Genotype	Hepatitis B e status			Total
	HBeAg + (%)	HBeAg-/Anti-HBe- (%)	HBeAg-/Anti-HBe+ (%)	
Wild Type	20 (60.6)	5 (38.5)	11 (32.4)	36
Mixed	13 (39.4)	6 (46.2)	15 (45.1)	34
Mutant	0	2 (15.4)	8 (23.5)	10
Total	33	13	34	80

One HBeAg negative case was excluded because the anti-HBe status was not known.

carried the pre-core mutant virus in association with the WT virus. On the other hand, the pre-core mutant was present both as the predominant virus (22.9%) as well as in association with the WT virus (43.8%) in the HBe negative samples. There was no apparent difference in the distribution of WT and MT virus strains between the HBe negative samples that were positive for HBV DNA by hybridization and those that were negative (Table 2). The distribution of the hepatitis B WT and pre-core mutant virus with the progress of the serological status from HBe positive through the 'e window' to the anti-HBe positive state is given in Table 3. The serological progression of the infection appears to be paralleled by an increase in the ratio of the mutant virus the wild type virus.

The nucleotide sequence of the WT viruses was examined to determine the genotype with respect to the nucleotide located at position 1858 (codon 15). Sequence information of 21 WT viruses (14 HBe positive and 7 HBe negative cases) were analyzed: 15 were found to carry T at 1858 (1858 CCT variant); the remaining 6 WT viruses carried a C at 1858 (1858 CCC variant). The frequency of the CCT variant in HBe positive cases was 85.7% (12 of 14 cases); the frequency in HBe negative cases was only 42.9% (3 of 7 cases).

It was noted that 16 out of 48 of the HBe minus cases were negative for the 1896G→A pre-core mutation. To ascertain the basis for the e minus phenotype in these cases, the preC/C promoter sequence of 8 of these samples was evaluated: five of the samples were found to carry the paired mutation (1762A→T and 1764 G→A) at the second AT rich region of the core promoter; one sample was found to carry a mutation at the pre-core start codon (1815T→C); in the remaining two cases, no mutations were found in either the core promoter or the pre-core region.

DISCUSSION

The study was conducted to provide information on the relative frequency of occurrence of the pre-core stop codon mutant in chronic hepatitis B carriers (including both HBe positive and negative cases). The results show that pre-core mutations resulting in loss of HBe expression are common among our chronic hepatitis B patients. HBV with the mutation is present in both HBe positive (39.4%) and HBe negative (66.7%) patients; in the case of the former, the mutation was found only in association with the wild type virus, as expected; in the HBe negative subjects, the mutation was present as the dominant virus population in 22.9% and as a mixed infection, in association with the WT virus, in 43.8% of cases.

There is an apparent increase in the fre-

quency of the pre-core mutant virus with progression of the serological status from HBe positive through the 'e-window' to HBe negative status (Table 3). This observation is consistent with the evolution of the infection in which chronicity is paralleled by the emergence of the mutant virus, which is followed by the failure of HBe antigen production and eventual HBe seroconversion. It is generally assumed that the emergence of the pre-core mutant occurs at the time of conversion from HBe antigen seropositivity to anti-HBe seropositivity. However, recent studies have questioned this relationship (Buckwold *et al*, 1996; Moriyama *et al*, 1996; Chan *et al*, 1999). Various investigators have demonstrated that conversion of the WT to the pre-core mutant status continued to occur for 2 to 3 years after HBe seroconversion, and that seroconversion and genome conversion may in fact be quite separate events (Maruyama *et al*, 1998; Chan *et al*, 1999); to confirm this, sequential follow-up genomic and serological studies are necessary.

The results of sequence analysis demonstrated that the HBV with 1858T (codon 15:CCT) is the dominant genotype among our isolates (85.7% in HBe positive cases). This is consistent with the frequent occurrence of the pre-core mutant virus in local isolates. Of the limited number of the 1858C variant virus examined in this study, none was found to have the 1896 G→A mutation (M1 mutant). This observation concurs with reports in geographical regions where the HBV genotype A, which has a variant proline codon of CCC at codon 15 is predominant, the 1896 G→A change is rare (Li *et al*, 1993; Rodriguez-Frias *et al*, 1995).

We noted that a third of the cases with the 'HBe minus' phenotype did not carry the pre-core mutation. Sequence data was available for a proportion of these cases (n=8): paired mutations at nucleotides 1762 and 1764 (1762A→T, 1764 G→A) residing within the second AT rich region of the core promoter were demonstrated in 5 of 8 cases; all 5 cases were WT variants with codon 15 CCC. The underlying basis of the HBe negative phenotype resulting from these two mutations has been described previously (Buckwold *et al*, 1996; Moriyama *et al*, 1996). In one additional case, the phenotype could be explained by a mutation in the pre-core start codon (1815T→C); we were unable to determine the basis for the phenotype in the two remaining cases-both had detectable HBV DNA by hybridization analysis (1 pg/200 μl and 200 pg/200 μl).

In summary, we were able to identify the mutations responsible for the e minus phenotype in 6 of the 8 pre-core mutant negative cases that were analyzed; projection of these data gives an estimated 90% detection rate for mutations responsible for the 'HBe minus' phenotype in our cases.

In conclusion, the pre-core point mutation (1896A) that results in the failure of HBe production is relatively common in our chronic hepatitis B patients. It is detectable both in HBe antigen positive and negative subjects, albeit at a lower frequency in the former. The frequent occurrence of the mutation is consistent with the predominant virus genotype in our isolates. Overall, 12 of the 14 (85.7%) HBe positive cases studied were found to be codon 15 CCT variants; the codon 15 CCC variant was also present, but at a much lower frequency - an observation noted in other studies involving Asian subjects. The predominant mutation that results in the HBe minus phenotype is the 1896A mutation. Other mutations identified included a double mutation at the second AT rich region of the pre-core/core promoter and a point mutation at nucleotide 1815, which results in the elimination of the pre-core start codon.

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