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Low pretreatment serum HBsAg level and viral mutations as predictors of response to PEG-interferon alpha-2b therapy in chronic hepatitis B☆

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ABSTRACT

Background: Viral genomic mutations have become increasingly recognized as being associated with the outcome of chronic HBV infection. However, the role of viral mutations as a predictor of response to pegylated-interferon (PEG-IFN) therapy has so far remained unclear.
Study design: Viral mutations in the enhancer II/basal core promoter (BCP)/precore and the pre-S regions were characterized by direct sequencing in pretreatment serum samples of 50 patients with chronic hepatitis B (33 HBeAg-positive and 17 HBeAg-negative), who were treated for 48 weeks with PEG-IFN alpha-2b.
Results: Sustained virological response at 48 weeks post treatment, defined as HBeAg seroconversion and HBV DNA < 2000 IU/mL for HBeAg-positive patients, and HBV DNA < 200 IU/mL for HBeAg-negative patients, was achieved in 12 (36.4%) and 6 (35.3%) of HBeAg-positive and HBeAg-negative patients, respectively. Response to PEG-IFN therapy correlated to low pretreatment HBsAg level but did not correlate with HBV genotype, pretreatment alanine transaminase and HBV DNA levels. In HBeAg-positive hepatitis, PEG-IFN response correlated with the appearance of double BCP mutations (A1762T/G1764A) at baseline ($P = 0.041$). In the HBeAg-negative group, response to PEG-IFN therapy was associated with the presence of pre-S mutation/deletions ($P = 0.028$). Multivariate analysis identified low pretreatment HBsAg level as an independent factor associated with SVR in both groups.
Conclusions: Pretreatment quantitative HBsAg determination is useful for predicting response to PEG-IFN therapy. The presence of double BCP and pre-S mutation/deletions at entry may be associated with a high rate of antiviral response in HBeAg-positive and HBeAg-negative hepatitis, respectively.

1. Introduction

Chronic hepatitis B virus (HBV) infection and its sequelae are major global health problems.[1] The currently approved agents for chronic hepatitis B are nucleoside/nucleotide analogues and interferon alfa (IFN)-based therapies.[2] IFN acts mainly as an immunomodulator and augments the cell-mediated immune response in the process of clearing the virus. The efficacy of conventional IFN therapy has been improved by using its pegylated form (peginterferon, PEG-IFN), resulting in higher response rates in patients with chronic hepatitis B. However, the overall sustained response rate to PEG-IFN can be achieved in only approximately one-third of patients.[3] Considering the fact that PEG-IFN treatment is expensive and could have some adverse effects, it is crucial to identify those patients for whom PEG-IFN treatment will be beneficial.

The effect of HBV mutations on the outcome of PEG-IFN therapy is largely unknown. The most well-known HBV variants include a precore (PC) stop codon mutation (G1896A), which abolishes hepatitis B e antigen (HBeAg) production, and double mutations in the basal core promoter (BCP) region (A1762T/G1764A), which downregulate HBeAg production. These mutations have been reported to influence the response to conventional IFN treatment.[4,5] Apart from these variants, other mutations such as T1753C/A/G in the BCP region and C1653T in the enhancer II region (EnhII) have become increasingly recognized as being associated with the outcome of chronic HBV infection.[6,7] Furthermore, naturally occurring mutations or deletions in the pre-S1/pre-S2 gene of HBV genome

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have been shown to frequently accumulate in chronically infected patients.[8,9] Whether these variants affect the response to PEG-IFN has been poorly investigated. Thus, the aim of this study was to determine pretreatment clinical and molecular virologic factors associated with response to PEG-IFN therapy in patients with chronic hepatitis B.

2. Materials and methods

2.1. Patients

Fifty patients with chronic hepatitis B (39 men and 11 women) who had completed the treatment with PEG-IFN alpha-2b (PEG-IFN-α2b; Shering-Plough, Kenillworth, NJ) and had been followed-up between August 2005 and January 2008 at King Chulalongkorn Memorial Hospital, Bangkok, Thailand were retrospectively investigated. The patients were aged between 22 and 61 years and fulfilled the following criteria: (1) sero-positive for hepatitis B surface antigen (HBsAg), elevation of serum alanine aminotrasferase (ALT) for at least 6 months and detectable serum HBV DNA; (2) no evidence of hepatocellular carcinoma (HCC) based on the clinical criteria and ultrasound examination at baseline; (3) no evidence of hepatitis C virus (HCV), hepatitis D virus (HDV) and human immunodeficiency virus (HIV) infection. Liver biopsy was performed within 6 months of entry. The liver histology was graded by the histological activity index (HAI) according to the criteria of Knodell et al.[10], which comprise two major components namely necroinflammation (HAI-I) and fibrosis (HAI-F). In this study, 33 and 17 patients were classified as HBeAg-positive and HBeAg-negative chronic hepatitis, respectively. Among these patients, 3 cases in the HBeAg-negative group were histological diagnosed of cirrhosis.

PEG-IFN-α2b was administered subcutaneously at a dose of 1.5 μg/kg weekly for 48 weeks. All patients were followed-up for up to 48 weeks after treatment (week 96) to assess sustained virological response (SVR). In the HBeAg-positive group, SVR was defined as HBeAg seroconversion and sustained inhibition of viral replication (HBV DNA level < 2000 IU/mL) until 12 months post treatment. In the HBeAg-negative group, SVR was defined as sustained inhibition of viral replication (HBV DNA level < 200 IU/mL) until 12 months post treatment.[11] Patients without SVR were defined as non-responders. According to these criteria, 12 (36.4%) and 6 (35.3%) of the HBeAg-positive and HBeAg-negative groups, respectively, were classified as responders.

Serum samples were collected from each patient at entry and stored at −70 °C until further tests were performed. All patients had been informed as to the study's purpose and had given their written consent. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

2.2. HBV DNA preparation, amplification, and direct sequencing

HBV DNA was extracted from 100 μL serum sample by incubation in lysis buffer (10 mM Tris–HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS and 20 mg/mL proteinase K) and phenol-chloroform-isoamyl alcohol extraction. The DNA pellet was resuspended in 30 μL sterile distilled water and subjected to amplification of the pre-S {nucleotides (nt) 2814–475} and EnhII/BCP/PC (nt 1552–2053) regions by polymerase chain reaction (PCR). The reaction mixture comprised 2 μL resuspended DNA, 0.5 μL of 25 mmol of each primer (pre-S region: Pre-S1F+ 5′-GG GTCACCATATTCTTGGGAAC-3′ and R3 5′-ACAAACGGGCAACATACCTTG-3′; EnhII/BCP/PC: X101 5′-TCTGTGCCTTCTCATCTG-3′ and CO2 5′-GTGAG GTGAACAATGTTCCG-3′), 10 μL of 2.5× MasterMix® (Eppendorf, Germany) and sterile distilled water to a final volume of 25 μL reaction. The reaction was performed in a PCR thermocycler (Eppendorf AG, Hamburg, Germany) with the initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s (denaturing), at 55 °C for 30 s (annealing), at 72 °C for 1 min (extension) and concluded by a final 7 min extension at 72 °C. The PCR products were segregated by 2% agarose gel electrophoresis. The PCR products were extracted from the agarose gel using the Perfectprep® Gel cleanup kit (Eppendorf, Hanburg, Germany). The sequencing reaction was performed using the AmpliTaq™ DNA Polymerase FS dye terminator from the ABIPRISM™ BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's specification. Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and aligned with CLUSTAL_X (version 1.83) program.

The obtained sequences were submitted to GenBank under accession numbers EU841367–EU841816 and EU850826–EU850874. The sequences were further characterized for the genotyping assay by phylogenetic analysis as previously described.[12]

2.3. Biochemical, serological and virological assays

Serum ALT level was measured with a commercial assay using an automated analyzer (Hitachi 912). Quantification of hepatitis B s antigen (HBsAg) was tested in pretreatment serum samples by ARCHITECT *i*2000SR (Abbott Diagnostic, Wiesbaden, Germany) according to the manufacturer's specifications.[13] The detection of the assay ranged from 0.05 to 250 IU/mL. Samples with HBsAg titers beyond the upper range were diluted with phosphate buffered saline (PBS) into 1:10, 1:1000 and 1:10,000 prior to further analysis. HBeAg and anti-HBe antibodies were determined using commercially available enzyme-linked immunosorbent assays (Abbott Laboratories, Chicago, IL). Serum HBV DNA level was quantified using a commercially available kit (Amplicor HBV Monitor; Roche Diagnostics, Tokyo, Japan). The detection of the assay ranged from 60 to 4×10^6 IU/mL

2.4. Statistical analysis

Data were expressed as mean ± standard deviation (SD), and percentages as appropriate. Comparisons between groups were analyzed by the χ^2 or Fisher's exact test for categorical variables and by the Mann–Whitney *U*-test or Student's *t*-test for quantitative variables. Multivariate analysis using stepwise logistic regression was performed to identify independent factors associated with PEG-IFN response. *P*-values < 0.05 were considered statistically significant. Data were analyzed by using the SPSS software for Windows 14.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Patient characteristics

Demographic and baseline clinical characteristics of the patients are shown in Table 1. HBeAg-negative patients had significantly higher mean age, but lower baseline HBV DNA and HBsAg levels than patients with HBeAg-positive hepatitis. No significant difference between groups was observed in respect to sex, pretreatment ALT level, necroinflammatory and fibrosis scores.

3.2. HBV genotype

Based on sequencing and phylogenetic analysis of the pre-S region, serum samples from 6 (12%) patients (all in the HBeAg-positive group) belonged to genotype B, and those of 44 (88%) (27 in the HBeAg-positive group) to genotype C (Fig. 1). Based on the

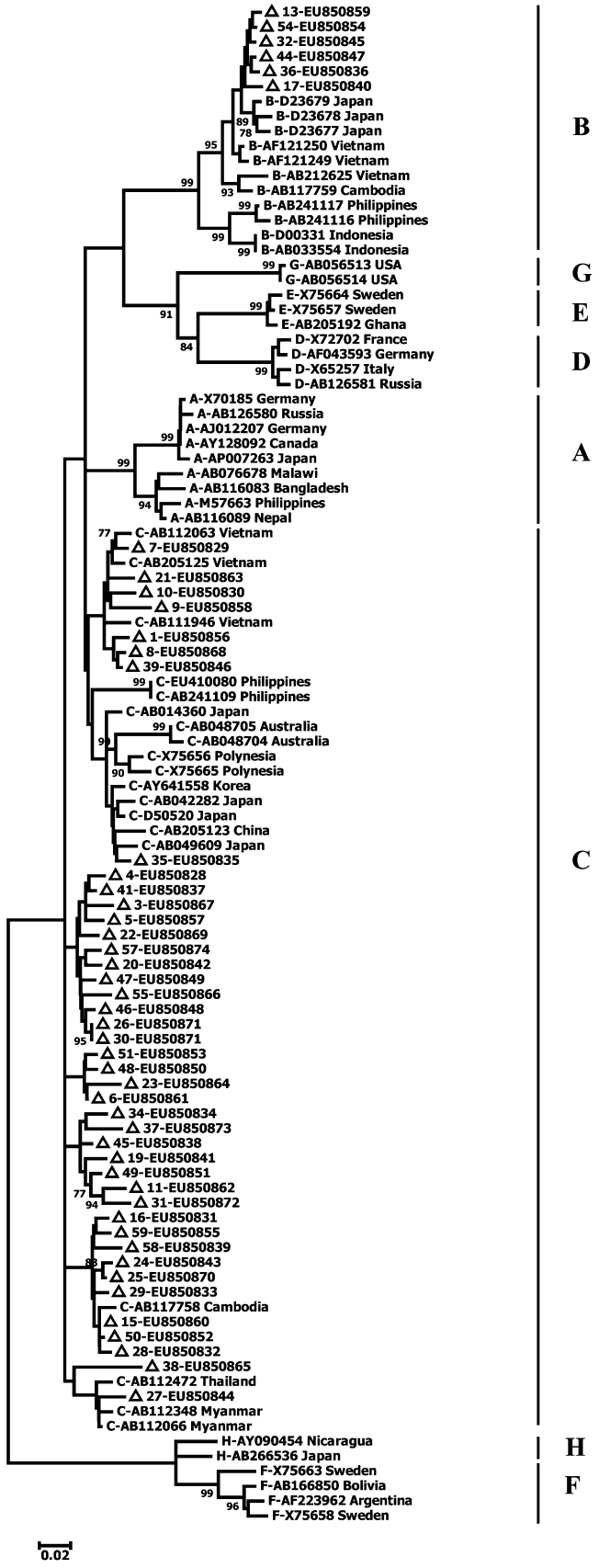


Fig. 1. Phylogenetic tree analysis of pre-S region obtained from sample sequences used in this study (EU841367–EU841816 and EU850826–EU850874).

Table 1
Demographic and clinical characteristics of HBeAg-positive and HBeAg-negative patients.

	HBeAg-positive ($n=33$)	HBeAg-negative ($n=17$)	P
Age, yr	37.4 ± 9.1	43.3 ± 8.0	0.023
Sex, male	26 (78.8%)	13 (76.5%)	1
Pretreatment ALT level, U/L	92.6 ± 84.4	102.6 ± 56.1	0.62
Pretreatment HBV DNA, $\log_{10}$ IU/mL	6.2 ± 0.8	5.7 ± 0.8	0.04
Pretreatment HBsAg level, IU/mL	14189.03 ± 19445.4	2668.5 ± 5037.4	0.003
Pretreatment HBsAg level, $\log_{10}$ IU/mL	3.9 ± 0.6	2.9 ± 0.8	<0.001
Necroinflammatory score	4.0 ± 1.6	4.3 ± 1.8	0.62
Fibrosis score	1.3 ± 0.9	2.0 ± 1.4	0.11
Genotype			0.061
B	6 (18.2%)	0 (0%)	
C	27 (81.8%)	17 (100%)	
C1653T	1 (3.0%)	4 (23.5%)	0.04
T1753C/deletion	6 (18.2%)	10 (58.8%)	0.003
Double BCP mutation			0.004
A1762T/G1764A	13 (39.4%)	15 (88.2%)	
Deletion	4 (12.1%)	1 (5.9%)	
G1896A	3 (9.1%)	7 (41.2%)	0.021
Pre-S mutation			0.1
Pre-S2 start codon	2 (6.1%)	3 (17.6%)	
Deletion	1 (3.0%)	2 (11.8%)	
Antiviral response	12 (36.4%)	6 (35.3%)	1

preC/C region results, all 50 cases belonged to genotype C and were determined as subgenotype Cs. Thus, all HBV strains of genotype B in this study were subgenotype B2, which is a recombinant with the preC/C region of genotype C. There was no significant difference in HBV genotype distribution between the HBeAg-positive and HBeAg-negative groups (Table 1).

3.3. HBV mutations in the EnhII/BCP/PC regions

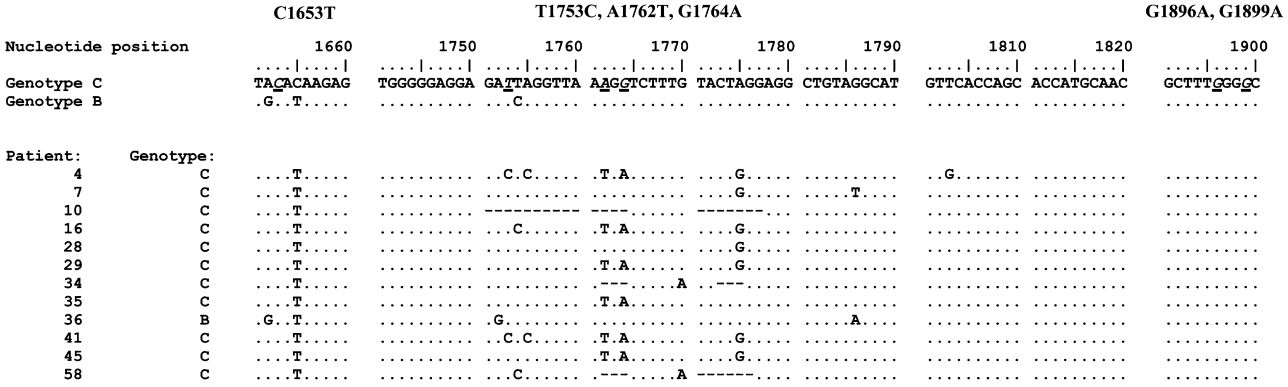
Mutational hot spots in these regions were found at nt 1753, 1762, 1764 and 1896. The C1653T and T1753C mutations were observed in 5 (10%) and 16 (32%) cases, respectively. The A1762T/G1764A mutations were found in 28 (56%) cases. In addition, five patients showed the following deletions at or around nt 1762–1764. One patient had deletions at nt 1762–1764 and 1773–1775, while another had deletions at nt 1762–1764 and 1771–1776. One additional patient had deletions at nt 1751–1764 and 1771–1777, while another one had a deletion at nt 1756–1770. All these cases with deletions belonged to the HBeAg-positive group. Furthermore, another patient in the HBeAg-negative group had a deletion at nt 1755–1763 and G1764A mutation (Fig. 2A and B). Comparison between groups showed that the frequencies of C1653T, T1753C, double BCP and PC mutations were significantly higher in HBeAg-negative patients (Table 1). In HBeAg-positive group, patients harboring double BCP mutations exhibited HBV DNA levels comparable to those with wild-type populations (6.8 ± 0.6 and 7.1 ± 0.9 $\log_{10}$ copies/mL, respectively, $P = 0.482$). Patients with HBV genotype C had higher frequencies of double BCP and T1753C mutations than those with HBV genotype B ($P < 0.001$ and $P = 0.023$, respectively). However, the frequencies of PC and C1653T mutations were not significantly different between genotypes B and C.

3.4. HBV mutations in the pre-S region

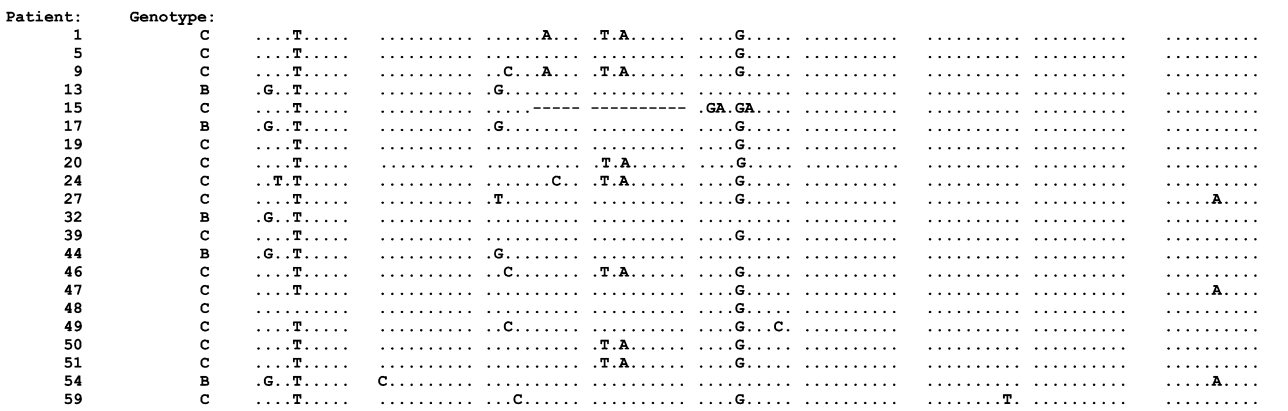
Pre-S mutations were detected in 8 of 50 (16%) samples. All these samples belonged to HBV genotype C, and were exclusively detected in viral populations harboring double BCP mutations.

(A) **HBeAg positive**

Sustained response

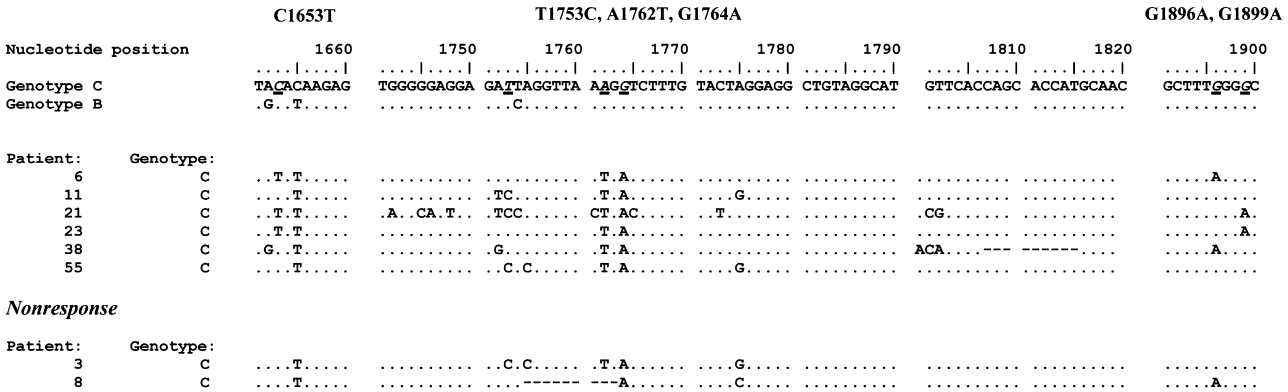


Nonresponse



(B) **HBeAg negative**

Sustained response



Nonresponse

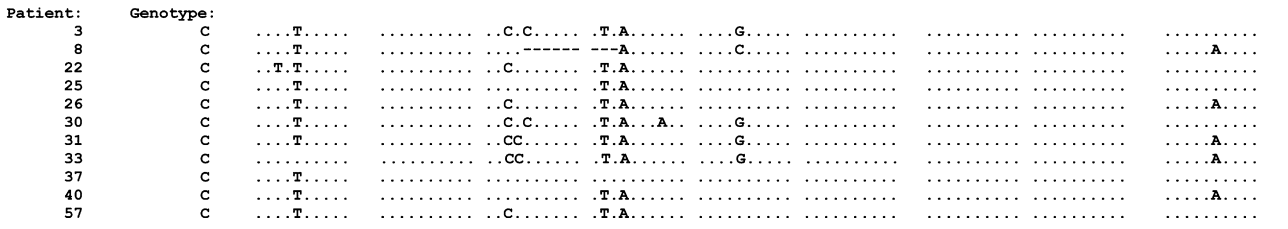


Fig. 2. The nucleotide sequences alignment of parts of the enhancer II/basal core promoter/precore regions. (A) HBeAg-positive patients and (B) HBeAg-negative patients. Nucleotide deletions are marked by a *hyphen* (-). Consensus sequence was established from HBV databank sequences.

Among these, pre-S2 start codon mutation was the most common (10%), followed by pre-S2 deletion (4%) and pre-S1 deletion (2%) (Fig. 3A and B). The prevalence of pre-S mutations was higher in the HBeAg-negative group but no significant difference was found (Table 1).

3.5. Factors associated with sustained response

In the HBeAg-positive group, low pretreatment HBsAg level and the presence of double BCP mutations at entry were found to be significant predictors of response to PEG-IFN therapy in

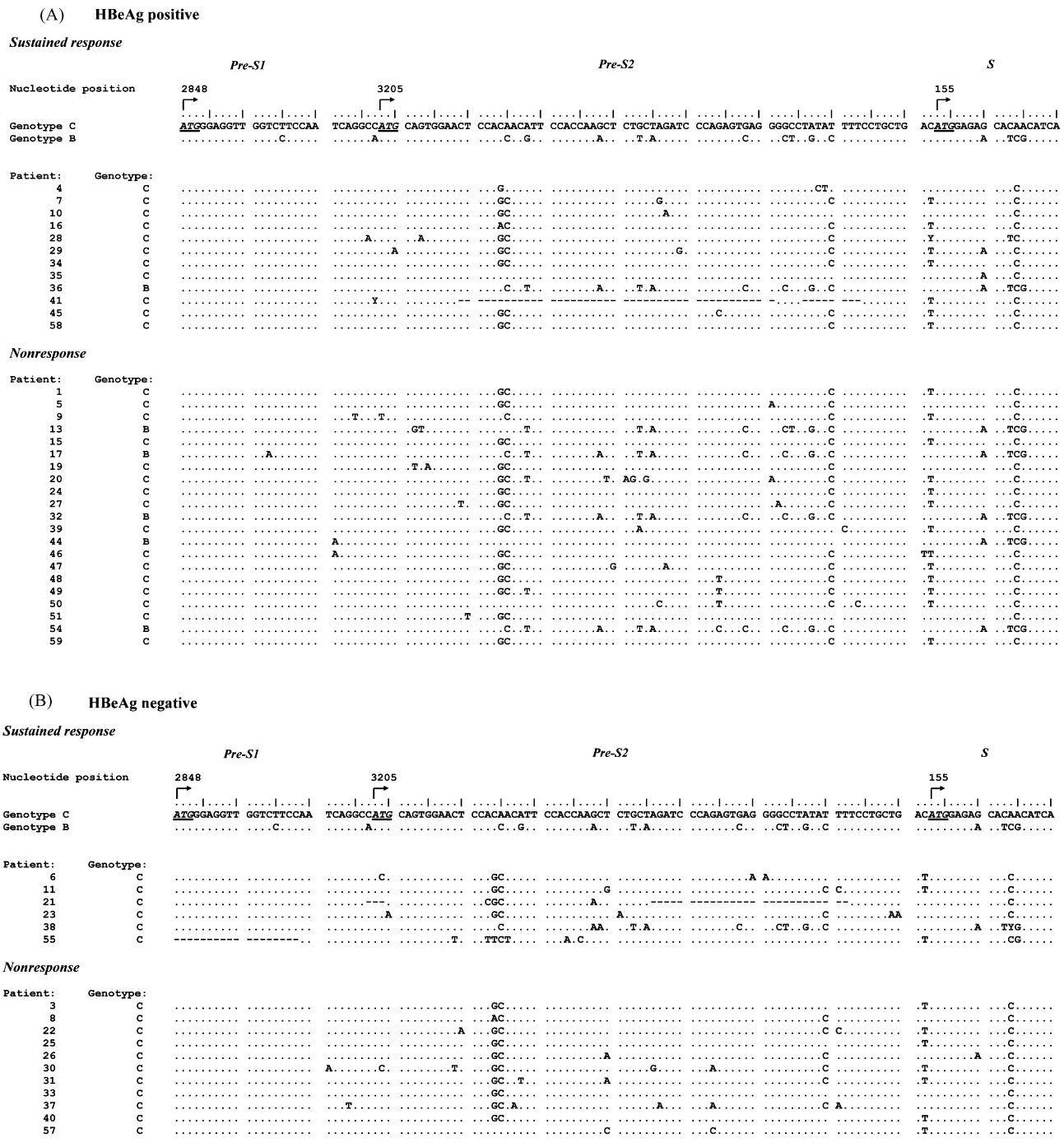


Fig. 3. The nucleotide sequences alignment of parts of the pre-S1/pre-S2 regions. (A) HBeAg-positive patients and (B) HBeAg-negative patients. Nucleotide deletions are marked by a *hyphen* (-). Consensus sequence was established from HBV databank sequences.

univariate analysis (Table 2). There was no correlation between the presence of C1653T, T1753C, G1896A, pre-S mutations and response to PEG-IFN treatment in this group. In the HBeAg-negative group, low pretreatment HBsAg level and presence of pre-S mutation/deletions at entry were found to be significant predictors of response (Table 3). However, there was no correlation between the presence of C1653T, T1753C, A1762T/G1764A, G1896A, and response to PEG-IFN treatment in this group. Multivariate analysis with stepwise logistic regression identified low pretreatment HBsAg level as an independent factor associated with sustained response in HBeAg-positive (odds ratio (OR), 14.67; 95% confidence interval (CI), 1.59–135.30; $P = 0.018$), and HBeAg-negative hepatitis (OR, 22.50; 95% CI, 1.61–314.56; $P = 0.021$).

4. Discussion

The results of this study showed that pretreatment C1653T and T1753C mutants were not associated with adverse response to PEG-IFN therapy. However, the presence of A1762T/G1764A at entry was associated with a higher rate of PEG-IFN response in HBeAg-positive patients. This is consistent with previous reports that HBeAg-positive patients harboring double BCP mutants responded better to conventional IFN therapy.[14–17] HBeAg, known to induce immunotolerance[18], has been used as a marker of infectivity and active viral replication. It has been shown that double BCP mutations downregulate precore mRNA transcription and decrease HBeAg production.[19] As a consequence, lower level of HBeAg could

Table 2
Baseline characteristics of HBeAg-positive patients with and without response to PEG-IFN therapy.

	Responder (n = 12)	Non-responder (n = 21)	P
Age, yr	38.9 ± 10.8	36.5 ± 8.2	0.51
Sex, male	8 (66.7%)	18 (85.7%)	0.19
Pretreatment ALT level, U/L	89.6 ± 52.2	94.2 ± 99.5	0.86
Pretreatment HBV DNA, $\log_{10}$ IU/mL	6.0 ± 0.5	6.4 ± 0.9	0.19
Pretreatment HBsAg level, IU/mL	4703.7 ± 3859.5	19,609.2 ± 22,626.9	0.007
Pretreatment HBsAg level, $\log_{10}$ IU/mL	3.5 ± 0.4	4.1 ± 0.6	0.005
Necroinflammatory score	3.6 ± 1.4	4.3 ± 1.7	0.23
Fibrosis score	1.3 ± 0.9	1.3 ± 1.0	0.81
Genotype			0.38
B	1 (8.3%)	5 (23.8%)	
C	11 (91.7%)	16 (76.2%)	
C1653T	0 (0%)	1 (4.8%)	1
T1753C/deletion	3 (25%)	3 (14.3%)	0.64
Double BCP mutation/deletion	9 (75%)	8 (38.1%)	0.041
G1896A	0 (0%)	3 (14.3%)	0.28
Pre-S mutation/deletion	2 (24%)	1 (4.8%)	0.54

Table 3
Baseline characteristics of HBeAg-negative patients with and without response to PEG-IFN therapy.

	Responder (n = 6)	Non-responder (n = 11)	P
Age, yr	47.0 ± 5.8	41.3 ± 8.5	0.12
Sex, male	5 (83.3%)	8 (72.7%)	1
Pretreatment ALT level, U/L	89.5 ± 44.1	109.7 ± 62.5	0.45
Pretreatment HBV DNA, $\log_{10}$ IU/mL	5.9 ± 0.8	5.6 ± 0.9	0.56
Pretreatment HBsAg level, IU/mL	346.4 ± 410.2	3935.1 ± 5959.6	0.014
Pretreatment HBsAg level, $\log_{10}$ IU/mL	2.3 ± 0.6	3.2 ± 0.6	0.008
Necroinflammatory score	5.3 ± 2.6	3.9 ± 1.4	0.39
Fibrosis score	3.0 ± 1.4	1.6 ± 1.3	0.15
Genotype			1
B	0 (0%)	0 (0%)	–
C	6 (100%)	11 (100%)	–
C1653T	3 (50%)	1 (9.1%)	0.09
T1753C/deletion	3 (50%)	7 (63.6%)	0.61
Double BCP mutation/deletion	6 (100%)	10 (90.9%)	1
G1896A	2 (33.3%)	5 (45.5%)	1
Pre-S mutation/deletion	4 (66.7%)	1 (9.1%)	0.028

contribute to a decrease in immunotolerance and enhance host immune response, which in turn lead to viral clearance following PEG-IFN treatment. In contrast, we could not find any correlation between the presence of PC mutant and PEG-IFN responsiveness. Indeed, there is no conclusive evidence that PC mutant has an impact on the outcome of IFN-based therapy.[14,15,20,21]

Although the role of pre-S mutants on the clinical course of chronic HBV infection has been recognized,[22] the role of these variants associated with PEG-IFN responsiveness has never been investigated. In HBeAg-negative patients, our data showed that pre-S mutation/deletions were observed more frequently in sustained responders, suggesting such variants may be associated with a higher response rate to PEG-IFN therapy. Although the reason is unclear, it is speculated that most of the mutation/deletion regions encompassed epitopes for T and B cells[23], which could lead to an alteration of the immune response and result in different outcome following PEG-IFN therapy. Interestingly, pre-S mutation/deletions were exclusively found in populations harboring double BCP mutations. Since both BCP and pre-S deletion mutants develop in the quasispecies during the course of persistent HBV infection, it is possible that the occurrence of these mutations are as a consequence of disease progression and may represent biomarkers for predicting the development of advanced liver disease.[8,24]

In this study, a low level of serum HBV DNA before treatment was not associated with response to PEG-IFN therapy. In contrast, low level of pretreatment HBsAg was consistently a predictor of the antiviral response in both HBeAg-positive and HBeAg-negative groups. This finding is in agreement with previous data that low pretreatment HBsAg levels have been considered as a predictor of HBsAg seroconversion following conventional IFN treatment.[25] It has been shown that low pretreatment HBsAg is more reliable than serum HBV DNA levels in predicting good response to PEG-IFN and lamivudine treatment.[26] Moreover, circulating HBsAg levels are strongly correlated with intrahepatic covalently closed circular DNA (cccDNA),[27] and cccDNA is superior to serum HBV DNA as a predictor of sustained response to antiviral therapy.[27,28] Thus, the present study provides additional evidence that quantitative HBsAg determination should be considered before administering IFN-based therapies.

The association between HBV genotypes and the response to PEG-IFN treatment remains controversial.[29,30] Our data showed that genotypes B and C were comparable in term of PEG IFN responsiveness. This finding could be in part attributed to the observation that all strains of genotype B were subgenotype Ba, which is a recombinant with the preC/C region of genotype C. Indeed, the epidemiologic study has demonstrated that all HBV strains in Thailand belong to subgenotype Ba.[31] Consequently, subgenotype Ba and genotype C, both of which share the sequences of the preC/C region, showed a similar response rate to PEG-IFN therapy. Whether there is any correlation between HBV genotypes/subgenotypes and the response to PEG-IFN therapy requires further investigation to address this interesting issue.

As this report has been one of the first studies to describe the molecular virological factors, particularly viral mutations and dele-

tions, that predict the response to PEG-IFN therapy, there have been some limitations. First, the number of patients included in this study was relatively small. Second, since HBV populations infecting patients are usually distributed as quasi-species,[32] variants are expected to coexist with wild-type strains in most cases. As viral mutations in this study were identified by direct sequencing of the PCR products without cloning, quantitative analysis for the relative amount of mutant or wild-type virus in mixed infection was not possible. As a result, data obtained in this study represented only predominant strains of HBV in the sera and minor viral variants could have escaped identification. Nonetheless, such minor strains might be less predictive of the response to PEG-IFN therapy than when they present as the predominant viral populations.

In conclusion, our data suggest that pretreatment quantitative HBsAg determination may be useful for predicting the response to PEG-IFN therapy and should be recommended in regular clinical practice. Double BCP mutations and pre-S mutations may also be associated with a high rate of antiviral response to PEG-IFN treatment in HBeAg-positive and HBeAg-negative hepatitis, respectively. Thus, analysis of these viral factors at baseline may lead to a better selection of patients for optimal PEG-IFN therapy. Further studies with larger sample sizes are required to verify these observations.

Acknowledgements

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