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Presence of Precore and Core Promoter Mutants Limits the Probability of Response to Peginterferon in Hepatitis B e Antigen-Positive Chronic Hepatitis B

Milan J. Sonneveld,[1] Vincent Rijckborst,[1] Stefan Zeuzem,[2] E. Jenny Heathcote,[3] Krzysztof Simon,[4] Hakan Senturk,[5] Suzan D. Pas,[6] Bettina E. Hansen,[1,7] and Harry L.A. Janssen[1]

Peginterferon (PEG-IFN) treatment of hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB) results in HBeAg loss in 30% of patients, but clearance of hepatitis B virus (HBV) DNA and hepatitis B surface antigen (HBsAg) from serum is less often achieved. We investigated whether the presence of precore (PC) and basal core promoter (BCP) mutants before PEG-IFN treatment affects serological and virological response. A total of 214 HBeAg-positive CHB patients treated with PEG-IFN±lamivudine for 52 weeks in a global randomized trial were classified at baseline as wildtype (WT) or non-WT (detectable mutants at PC/BCP) by line-probe assay. Response was assessed at 6 months posttreatment and through long-term follow-up (LTFU). Mutants were detected in 64% of patients, in varying frequencies across HBV genotypes A through D. Patients with WT had higher baseline HBV DNA, HBeAg, and HBsAg levels than patients with non-WT. Patients with WT were more likely to achieve HBeAg loss with HBV DNA <10,000 copies/mL (response, 34 versus 11%, $P < 0.001$) and HBsAg clearance (18 versus 2%, $P < 0.001$) at week 78 than non-WT patients. Among WT patients who achieved HBeAg clearance at week 78, 78% had undetectable HBV DNA and 61% achieved HBsAg clearance at LTFU (versus 26% and 15% in non-WT patients, $P < 0.001$ for both). The presence of WT virus at baseline was an independent predictor of response (odds ratio [OR] 2.90, 95% confidence interval [CI]: 1.15-7.31, $P = 0.023$) and HBsAg clearance (OR 5.58, 95% CI: 1.26-24.63, $P = 0.013$) and patients with non-A genotypes with detectable mutants had a low probability of response. *Conclusion:* The presence of only WT virus at baseline is a strong predictor of response (HBeAg loss with HBV DNA <10,000 copies/mL) to PEG-IFN for HBeAg-positive CHB. Patients with detectable PC and/or BCP mutants have a lower probability of response and are less optimal candidates for PEG-IFN therapy. (HEPATOLOGY 2012;00:000–000)

Hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB) is generally regarded as the earliest stage of a four-stage disease continuum.[1-3] HBeAg does not appear to be required for infection with hepatitis B virus (HBV), nor for viral replication, but the presence of HBeAg in serum is associated with higher HBV DNA levels and was also recently shown to be an independent risk factor for the development of hepatocellular carcinoma (HCC).[4,5] Clearance of HBeAg (with or without the appearance of anti-HBe) has therefore been adopted as a primary treatment endpoint for HBeAg-positive CHB.[1,3]

Current treatment options for CHB result in increased rates of HBeAg clearance when compared with placebo-treated patients. A 1-year course of

Abbreviations: ALT, alanine aminotransferase; AUC, area under the receiver-operating characteristic curve; CHB, chronic hepatitis B; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PEG-IFN, peginterferon; ULN, upper limit of normal.

From the [1]Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands; [2]Medical Clinic 1, Johann Wolfgang Goethe University Medical Center, Frankfurt, Germany; [3]Division of Gastroenterology, University of Toronto, Toronto, Canada; [4]Department of Infectious Disease, Medical University Wroclaw, Wroclaw, Poland; [5]Department of Gastroenterology and Hepatology, Bezm-i Alem University, Istanbul, Turkey; [6]Department of Virology, Erasmus MC University Medical Center, Rotterdam, The Netherlands; [7]Department of Biostatistics, Erasmus MC University Medical Center, Rotterdam, The Netherlands.

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Address reprint requests to: Prof. Harry L.A. Janssen, M.D., Ph.D., Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, 's Gravendijkwal 230, room Ha 204, 3015 CE Rotterdam, The Netherlands. E-mail: h.janssen@erasmusmc.nl; fax: +31 (0) 10 436 5916.

peginterferon (PEG-IFN) results in HBeAg clearance in 34% of patients, compared with about 20% in patients treated with lamivudine (LAM), entecavir, or tenofovir.[1,6] However, emerging data show that the mere loss of HBeAg from serum may be insufficient to induce disease remission. Indeed, among patients who experience HBeAg clearance during nucleo(s)tide analog (NA)-based therapy, a considerable number experience HBeAg reversion or have persistently detectable HBV DNA levels after discontinuation of therapy.[7] Similarly, whereas HBeAg loss induced by IFN therapy seems to be more durable, a proportion of patients continue to have detectable HBV DNA during long-term follow-up (LTFU).[8-11] A possible explanation for these observations is the presence of viral strains that have mutations in the precore (PC) and basal core promoter (BCP) regions that prohibit the synthesis of HBeAg. The presence of these mutants before treatment initiation has been shown to predict HBeAg loss after IFN treatment,[12] possibly through positive selection during antiviral therapy,[13] but may predispose patients to persistence of HBV DNA after HBeAg clearance.[14,15]

The aims of the current study were therefore to study the presence and frequency of PC and BCP mutants within a cohort of HBeAg-positive CHB patients and to relate the presence of PC and BCP mutant strains to serological and virological response to PEG-IFN therapy.

Patients and Methods

Patients. In this study the presence of PC and BCP mutants was assessed in a cohort of HBeAg-positive CHB patients who were previously enrolled in an investigator-initiated multicenter randomized trial and had available hepatitis B surface antigen (HBsAg) quantification measurements.[6,8,16] The inclusion and exclusion criteria for the original study have been described elsewhere.[6] In summary, patients were eligible if they had been HBsAg-positive for at least 6 months before randomization, were HBeAg-positive, had elevated serum alanine aminotransferase (ALT) levels of >2, but <10 times the upper limit of normal (ULN), and had a serum HBV DNA concentration of more than 1.0×10^5 copies/mL. Patients were treated with PEG-IFN alpha-2b 100 μg weekly (PegIntron, Schering-Plough, Kenilworth, NJ) in combination with placebo or LAM 100 mg (Zeffix, GlaxoSmithKline, Greenford, UK) daily for 52 weeks. The dose of PEG-IFN was reduced to 50 μg per week after 32 weeks of treatment to limit the probability of treatment discontinuation.

Inclusion criteria for the present analysis were completion of the 26-week follow-up phase of the main study and availability of a baseline serum sample for PC/BCP mutation assessment. Of the 266 patients in the initial study, 214 fulfilled these criteria.

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. All patients gave written informed consent according to standards of the local Ethics Committees.

Laboratory Measurements. The presence of PC and BCP mutants was assessed using the INNO-LiPA HBV PreCore assay (Innogenetics, Ghent, Belgium). This very sensitive line probe assay allows for easy detection of PC (at nucleotide position G1896) and BCP (at nucleotide positions A1762 and G1764) mutants, even when only present as minority species.[17] Patients were subsequently classified as wildtype (WT, only WT virus detectable), PC (only PC or both PC and WT detectable), BCP (either or both BCP mutations detected, with or without WT), or as both when PC and BCP mutants were found. Serum HBV DNA, HBeAg, and HBsAg was quantified in samples taken at baseline, during the treatment period (weeks 4, 8, 12, 24, and 52), and during follow-up (week 78). HBV DNA quantification was performed using an in-house developed TaqMan polymerase chain reaction (PCR) assay (lower limit of quantification 400 copies/mL) based on the EuroHep standard.[18] HBsAg was measured using the Abbott ARCHITECT HBsAg assay (Abbott Laboratories; range 0.05-250 IU/mL) and HBeAg with the Roche ELECSYS HBeAg assay using a quantitative protocol (Roche Diagnostics, range 0.2-100 IU/mL).

Statistical Analysis. For the current study a composite endpoint of HBeAg loss and HBV DNA level <10,000 copies/mL (≈2,000 IU/mL) was chosen for

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Table 1. Characteristics of the Study Cohort

Characteristics	Study Population (n=214)
Demography	
Mean (SD) age, years	33.8 (13)
Male	167 (78%)
PEG-IFN monotherapy	104 (49%)
Race	
Caucasian	157 (73%)
Asian	40 (19%)
Other	17 (8%)
Laboratory results	
Mean (SD) ALT*	4.3 (3.0)
Mean (SD) HBV DNA, log c/mL	9.1 (0.90)
Mean (SD) HBsAg, log IU/mL	4.4 (0.60)
Mean (SD) HBeAg, log IU/mL	2.5 (0.70)
HBV genotype	
A	74 (35%)
B	19 (9%)
C	29 (14%)
D	85 (40%)
Other/mixed	7 (3%)
INNO-LiPA result	
Wildtype	76 (36%)
Precore	56 (26%)
Basal core promoter	47 (22%)
Precore and basal core	35 (16%)
Response at week 78	
Response†	41 (19%)
HBeAg loss	77 (36%)
HBV DNA <10,000 c/mL	43 (20%)
HBsAg loss	17 (8%)

*Multiples of upper limit of the normal range.
†HBeAg loss and HBV DNA <10,000 copies/mL at week 78.

definition of response at week 78, because this endpoint is associated with a low probability of relapse and low risk of disease progression.[5,19,20] At LTFU, retreated patients were classified as nonresponders. For multivariate analyses, patients were classified as WT (only WT virus detectable) or non-WT (detectable PC and/or BCP mutants). Associations between variables were tested using Student's *t* test, chi-square, Pearson correlation, or their nonparametric equivalents when appropriate. To investigate the value of WT/non-WT status in addition to our previously published PEG-IFN Treatment Index prediction model,[19] we used a multivariate logistic regression model with the predicted probability calculated using the PEG-IFN Treatment Index as a fixed value. The PEG-IFN Treatment Index was built using data on HBV genotype, baseline levels of ALT and HBV DNA, as well as age, patient gender, and previous IFN therapy. The gain of adding WT/non-WT status was quantified using the net reclassification improvement (NRI), which is the sum of reclassification of subjects with a response and without a response, where reclassification is the percentage of patients with an improved prediction minus the percentage of patients with a worse prediction.[21] SPSS v. 15.0 (Chicago, IL) and the SAS 9.2 program (Cary, NC) were used to perform statistical analyses. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

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Results

Patient Characteristics. Because treatment outcome did not differ between patients treated with PEG-IFN ± LAM, the data of the two treatment arms were pooled for the current analysis. The characteristics of the 214 patients are shown in Table 1.

Prevalence of PC and BCP Mutants at Baseline. Within the total cohort, a minority (36%) of patients had only WT virus detectable. PC and BCP mutants were detected in 138 (64%) of patients, either alone or in combination (Table 1). When stratified by HBV genotype, considerable differences were observed with regard to the frequency and type of detected mutant virus ($P < 0.001$). A majority of patients with genotype A harbored only WT virus, whereas mutants were often detected in patients with genotypes B, C, and D (Fig. 1).

Relationship Between PC and BCP Mutants and HBV Serum Markers at Baseline. Considerable differences were observed with regard to baseline HBV

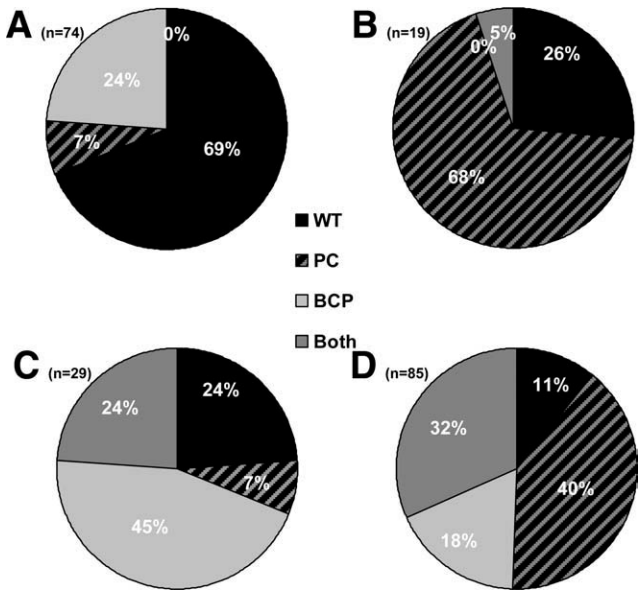

Fig. 1. Frequency of PC and BCP mutants in the cohort by HBV genotype.

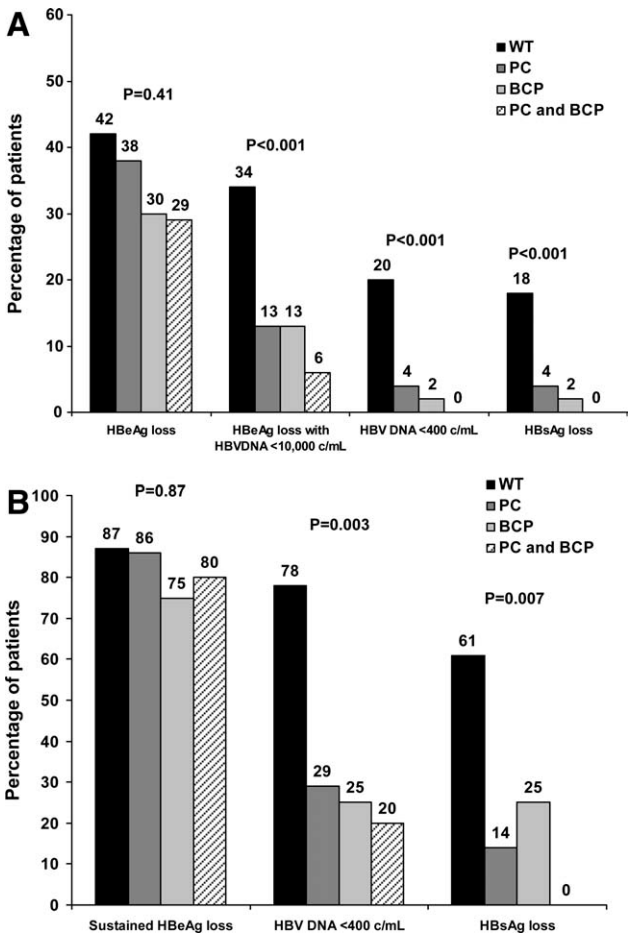

Fig. 2. Relationship between the presence of PC and BCP mutants at baseline and response at week 78 in the total cohort (n = 214) (A) and response at long-term (mean 3.0 years) follow-up among patients who were HBeAg-negative at week 78 (n = 50) (B). Response was defined as HBeAg clearance and HBV DNA <10,000 copies/mL at week 78. WT, wildtype; PC, precore; BCP, basal core promoter.

DNA, HBeAg, and HBsAg levels in patients with WT or detectable PC or BCP mutants. After adjustment for HBV genotype distribution, patients with WT had higher baseline HBV DNA levels (9.20 versus 8.86 log copies/mL, $P = 0.015$), higher HBeAg levels (2.81 versus 2.33 log IU/mL, $P < 0.001$), and higher HBsAg levels (4.53 versus 4.28 log IU/mL, $P = 0.007$) than patients with detectable mutants.

Relationship Between PC and BCP Mutants and Treatment Response at Week 78. Among the total population, rates of HBeAg loss were not significantly different between patients with WT virus or PC/BCP mutants (Fig. 2A). However, patients with only WT virus were considerably more likely to achieve a response (HBeAg loss and HBV DNA <10,000 copies/mL; 34 versus 11%, $P < 0.001$), HBV DNA <10,000 copies/mL (34% versus 12%, $P < 0.001$), HBV DNA <400 copies/mL (20% versus 2%, $P < 0.001$), and HBsAg clearance (18 versus 2%, $P < 0.001$) by week 78, when compared with patients with PC/BCP mutants. Patients with both PC and BCP mutants had both a very low probability of response and no chance of HBsAg loss.

LTFU of Patients with Negative HBeAg at Week 78. LTFU (mean 3.0 years, range 1.9-5.0) data were available in 50 of 77 (65%) patients who were HBeAg-negative at week 78. The majority of patients had sustained HBeAg loss through prolonged follow-up, irrespective of the presence of WT or PC/BCP mutants at baseline (Fig. 2B). However, patients with only WT virus at baseline who cleared HBeAg by week 78 had a much higher probability of achieving undetectable (<400 copies/mL) HBV DNA levels at LTFU when compared with those with mutants ($P = 0.003$, Fig. 2B). Additionally, patients who achieved HBeAg loss at week 78 and had WT virus at baseline had a 61% probability of being HBsAg-negative at LTFU.

Presence of PC and BCP Mutants in Patients with HBeAg Loss with HBV DNA >10,000 Copies/mL at Week 78. Of the 36 patients who cleared HBeAg but had HBV DNA >10,000 copies/mL, serum samples and PC/BCP data at week 78 were available in a subset of 29 (81%). All of the patients with detectable mutants at baseline had detectable mutants at week 78, and five out of six patients with WT at baseline had detectable mutants at week 78 (Fig. 3).

Relationship Between PC/BCP Mutants and Response; Multivariate Analysis. To account for the differences in PC and BCP mutant distributions across HBV genotypes, the presence of WT/non-WT status was added as an independent determinant of response to other previously described predictors, HBV genotype, baseline HBV DNA level, ALT, and age. The presence of only WT virus remained a strong predictor of response (odds ratio [OR] WT versus non-WT: 2.60, 95% confidence interval [CI]: 1.05-6.42, $P = 0.037$). Combination therapy (PEG-IFN + LAM) was not associated with response. Similarly, the presence of only WT virus at baseline was a significant predictor of HBsAg loss at week 78 (OR for WT versus non-WT: 5.58, 95% CI: 1.26-24.63, $P = 0.013$), after adjustment for other predictors, HBV genotype, and age. Flowcharts for treatment response for WT versus non-WT by HBV genotype and baseline HBV DNA and ALT levels are depicted in Fig. 4A,B.

We added WT/non-WT status to a model including the previously published PEG-IFN treatment index[19] and found that it significantly improved prediction of

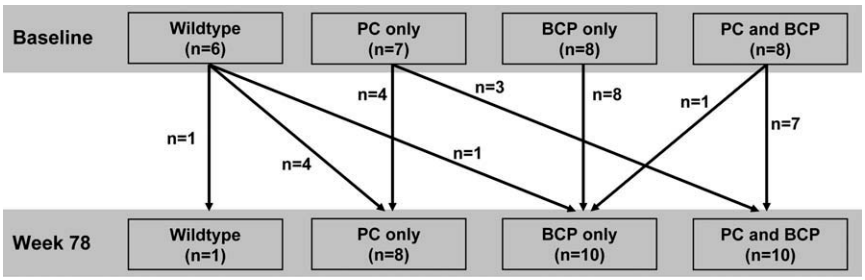

Fig. 3. Baseline and week 78 INNO-LiPA test results in patients with HBeAg loss and HBV DNA >10,000 copies/mL (n = 29). WT, wildtype; PC, precore; BCP, basal core promoter.

response (WT versus non-WT OR 2.35, 95% CI: 1.06-5.19, $P = 0.033$, NRI = 0.66). The individual patients' estimated probabilities for the PEG-IFN Treatment Index extended with WT/non-WT status are depicted in Fig. 4C. Recalibration of the model showed that WT/non-WT significantly predicts response to PEG-IFN, independently of HBV genotype, baseline ALT and HBV DNA, patient age, and previous IFN therapy failure (Table 2). An interaction term between HBV genotype and WT/non-WT was not significant ($P = 0.954$). Figure 5A shows the estimated probabilities of response from this model for WT/non-WT, stratified by HBV genotype. Figure 5B shows the estimated probabilities of HBsAg clearance for patients with WT or non-WT, stratified by HBV genotype and adjusted for patient age.

PC and BCP Mutants and On-Treatment HBV DNA, HBeAg, and HBsAg Levels. To explore the relationship between presence of PC/BCP mutants and on-treatment kinetics of HBV DNA, HBeAg, and HBsAg, only data from the PEG-IFN monotherapy group were analyzed, because HBV DNA and HBsAg kinetics differ considerably between patients treated with PEG-IFN monotherapy and PEG-IFN + LAM combination therapy.[6,16] Within the monotherapy cohort, 40 (38%) patients harbored only WT virus (WT group), and 64 (62%) had evidence of either PC mutants, BCP mutants, or both (non-WT group).

HBV DNA. After 52 weeks of treatment, HBV DNA decline from baseline was 2.49 log copies/mL in the WT group, compared with 2.22 log copies/mL in patients with non-WT ($P = 0.60$). After treatment discontinuation, relapse was observed in patients with mutants, whereas patients with only WT virus at baseline continued to decline. Mean HBV DNA declines at week 78 were 2.82 versus 1.77 log copies/mL ($P = 0.05$, Fig. 6A).

Quantitative HBeAg. HBeAg decline was somewhat more pronounced during the first 8 weeks of therapy in patients with PC or BCP mutants, but end of treatment HBeAg declines were similar in both

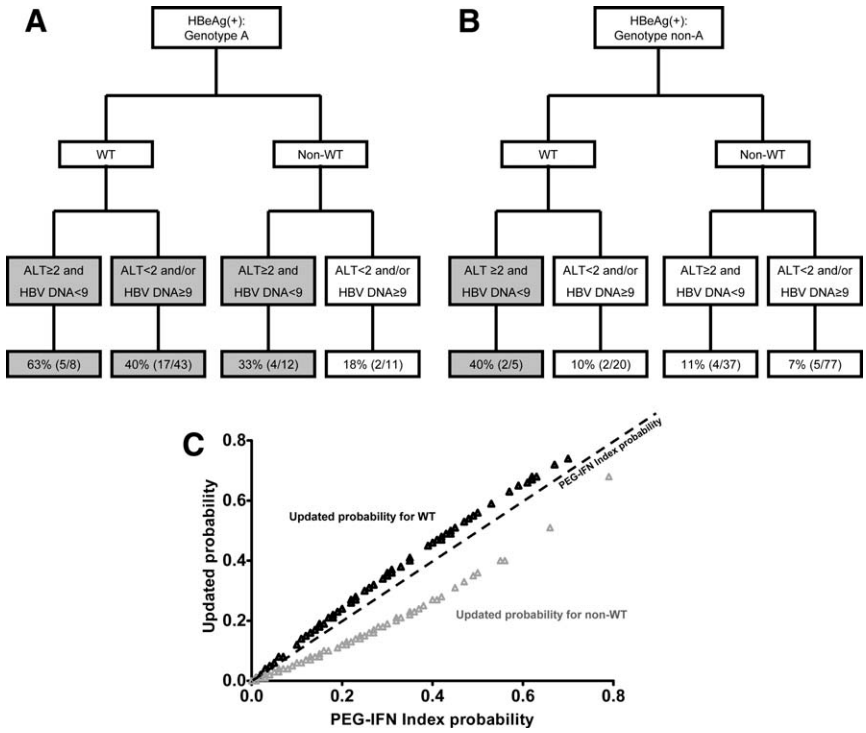

Fig. 4. Probability of response (HBeAg loss with HBV DNA <10,000 copies/mL at week 78) according to the presence of WT or detectable mutants (non-WT) and baseline HBV DNA and ALT levels, stratified by genotype A (A) or non-A (B). Gray boxes mark patient groups where PEG-IFN should be considered based on a probability of more than 30%.[19] (C) The individual probability of response as estimated by extending the previously published PEG-IFN Treatment Index[19] with data on WT/non-WT. WT, wildtype.

Table 2. Logistic Regression Model of Probability of Response to Peginterferon

Variable	OR (95% CI)	P
WT	2.90 (1.15-7.31)	0.023
HBV genotype		0.043
A	Ref.	
B	0.56 (0.14-2.21)	
C	0.11 (0.02-0.59)	
D	0.35 (0.11-1.14)	
HBV DNA*	0.58 (0.35-0.97)	0.038
ALT†	1.10 (0.95-1.26)	0.210
Age	1.04 (1.01-1.08)	0.014
No previous IFN	5.20 (1.55-17.4)	0.003

*HBV DNA in log copies/mL.
†ALT in x ULN.

groups: 1.07 versus 1.04 log IU/mL in patients with only WT or detectable mutants, respectively ($P = 0.93$), as were HBeAg declines at week 78 ($P = 0.94$, Fig. 6B).

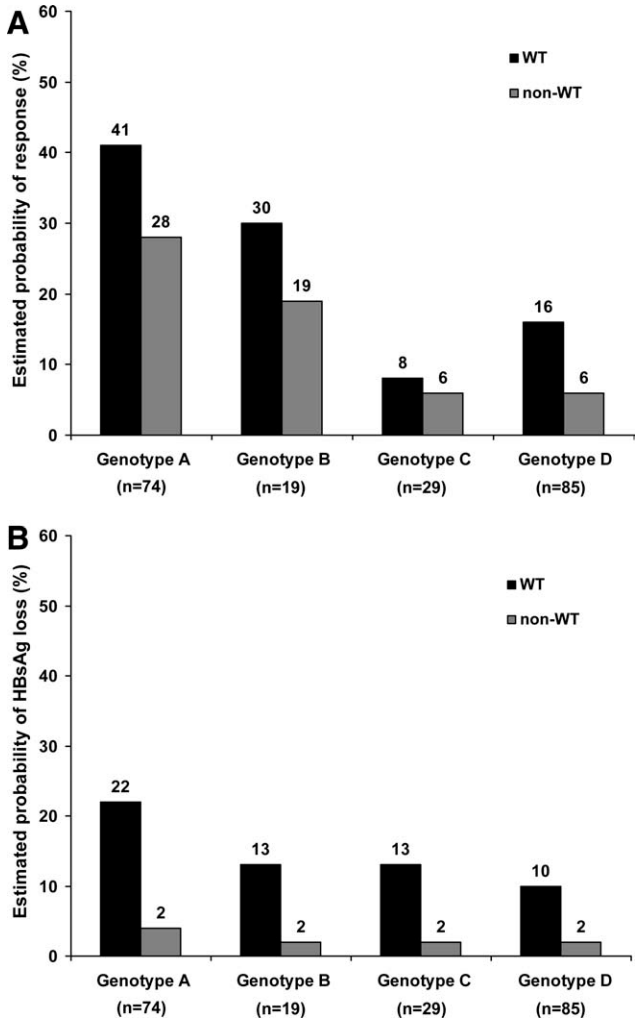

Fig. 5. The estimated probability of response from the model shown in Table 2 for WT versus non-WT, stratified by HBV genotype (A), and the estimated probability of HBsAg loss at week 78 by WT/non-WT, adjusted for patient age and stratified by HBV genotype (B).

Quantitative HBsAg. Patients with only WT virus detectable before therapy had a distinctly steeper HBsAg decline than did patients with PC or BCP mutants. End of treatment declines were 1.46 and 0.43 log IU/mL ($P = 0.007$), and the difference increased through off-treatment follow-up to 1.55 versus 0.38 log IU/mL at week 78 ($P = 0.003$, Fig. 6C).

Discussion

This is the largest study to date investigating the relationship between the presence of PC and BCP mutants and response to PEG-IFN in HBeAg-positive

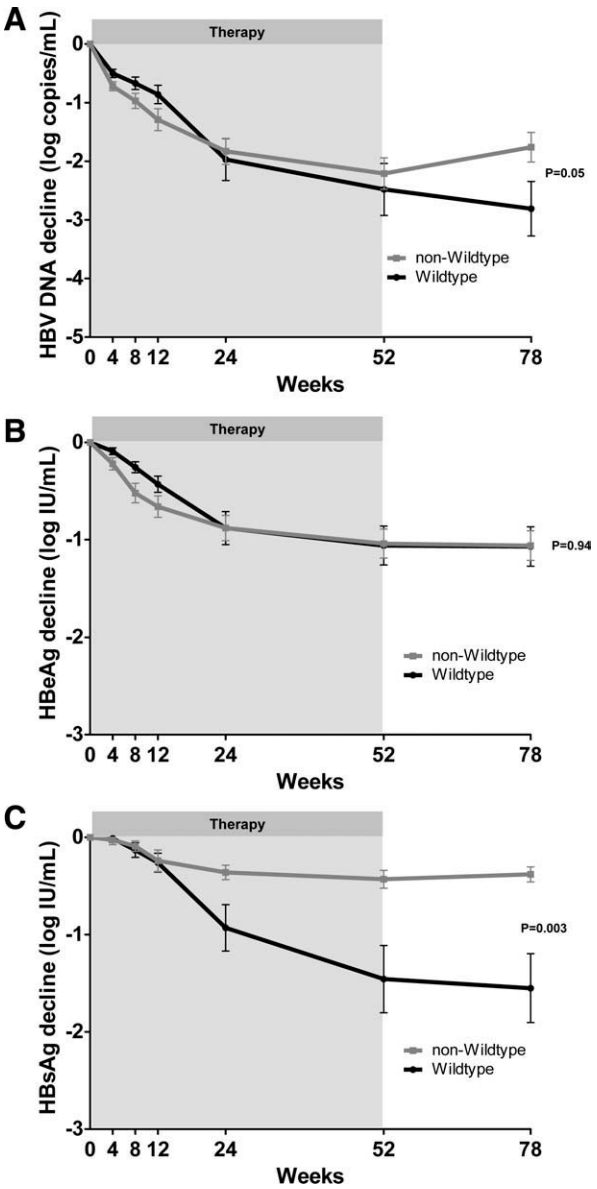

Fig. 6. HBV DNA (A), HBeAg (B), and HBsAg (C) declines among patients with only WT virus, or with detectable PC or BCP mutants (non-WT). Analyses were performed in patients treated with PEG-IFN monotherapy (n = 104). WT, wildtype.

CHB patients. We found that achievement of combined serological and virological response, as well as HBsAg seroclearance, is largely confined to patients without detectable PC and BCP mutants at baseline. Assessment of WT/non-WT status before therapy thus provides valuable additional insight into an individual patient's probability of achieving a response to PEG-IFN, and can help optimize patient selection for this burdensome treatment modality.

The prolonged presence of HBeAg in serum of CHB patients was recently shown to be an independent predictor of emergence of HCC, and clearance of HBeAg, whether spontaneous or treatment-induced, and is therefore considered an important event.[1,3] However, it has become increasingly clear that a considerable number of patients with CHB have persistently detectable HBV DNA levels after HBeAg clearance, predisposing these patients to progression of their liver disease to cirrhosis, HCC, and premature death.[5,22,23] A possible mechanism for this phenomenon is the selection for HBV mutants with mutations in the PC or BCP region, both of which reduce or abolish the production of HBeAg and are able to maintain replication despite seroclearance of HBeAg.[14,24] Data from the current study now show that these PC and BCP mutants may be detected in the majority of HBeAg-positive patients, confirming data from a U.S. survey.[25] The clinical relevance of these findings, especially in relation to response to immunomodulatory treatment of CHB, was, however, unclear. In patients treated with NA, the presence of PC or BCP mutants confers an increased probability of HBeAg clearance.[26] Similarly, several older studies have reported that the presence of PC or BCP mutants is associated with a higher probability of achieving response to standard IFN treatment.[12,27,28] Response in these studies was commonly defined as HBeAg clearance, with or without an HBV DNA undetectability criterion. However, most of these studies used HBV DNA assays that were not sensitive enough to detect HBV DNA at levels that are currently considered to confer substantial risk for progression of liver disease.[23] In concurrence with these studies, we found that patients with PC or BCP mutants have a good probability of HBeAg clearance, and of remaining HBeAg-negative through prolonged follow-up. However, the current study also shows that the majority of patients with detectable PC or BCP mutants who achieve HBeAg clearance do not achieve HBV DNA levels <10,000 copies/mL, necessitating further therapy in these patients according to recent guidelines.[1,3] Furthermore, it is important to note that HBeAg clearance alone does not appear to be an appropriate marker for immune control in patients with detectable PC or BCP mutants at baseline because HBsAg loss after HBeAg clearance is extremely rare in this group of patients. These observations, along with the high frequency (>60%) of PC and BCP mutant strains in our HBeAg-positive cohort, suggest that HBeAg clearance alone is not a suitable marker for response to PEG-IFN therapy in the general HBeAg-positive patient population, but perhaps only in those with confirmed WT virus at baseline.

We observed considerable differences in the frequency of PC and BCP mutants across HBV genotypes A through D. These observations are in line with other reports, and may be accounted for by differences in the genetic makeup of the respective genotypes.[25] Several recent reports, also from our group, have shown that the HBV genotype is an important predictor of response to (PEG-)IFN-based therapy of CHB.[19] The current study sheds considerable new light on these observations, because the advantages of patients with genotype A may be partly due to the relatively high frequency of WT virus, accounting for the high rates of HBV DNA undetectability and HBsAg loss through long-term follow-up in patients with HBV genotype A.[8] Nevertheless, the advantages of patients with only WT virus over those with detectable PC or BCP mutants holds true independent of HBV genotype. The presence of only WT virus is a very strong independent predictor of response and HBsAg clearance, when adjusting for the other established predictors HBV genotype, baseline HBV DNA level and ALT level, and age. Importantly, WT/non-WT status adds significantly to our previously published PEG-IFN Treatment Index prediction model.[19] Extension of this model with data on detection of mutants may help optimize prediction of response at baseline and help select only those patients who have a very high probability of response for PEG-IFN therapy. Moreover, detection of PC or BCP mutants in patients with non-A genotypes confers such a low probability of a combined HBeAg and HBV DNA response that in our view PEG-IFN should not be used as a first-line treatment option in these patients.

Another important observation is the relationship between the presence of PC and BCP mutants and established markers of HBV infection such as HBV DNA, HBeAg, and HBsAg. We found comparable on-treatment HBV DNA and HBeAg declines in patients with only WT or with mutant virus. These findings show the inherent limitations of HBeAg levels in monitoring PEG-IFN therapy efficacy, for they are highly influenced by the presence of PC and BCP mutants

and underscore the limitations of recent prediction rules for PEG-IFN therapy based on HBeAg thresholds.[29] In contrast, the current study confirms recent data on the relationship between HBsAg decline during PEG-IFN therapy and sustained off-treatment response, for HBsAg decline was largely confined to patients with WT virus.[16,30]

Possible limitations of the current study are related to the assay used to classify patients as having mutant virus. The INNO-LiPA assay is more sensitive than conventional sequencing technology and can detect mutant virus at very low levels.[17] However, the prevalence of mutants at baseline may have been underestimated if mutants were only present as a minority species (<5%).[31] Additionally, the assay can only detect known mutations and ignores others. Possible misclassification introduced by this limitation may have influenced the results. The current study enrolled patients infected with all major HBV genotypes, but only a limited subgroup of patients was infected with genotypes B and C. Possibly, this may have resulted in a nonsignificant test for interaction of WT with HBV genotype. Confirmation of our findings in Asian patients is therefore required.

In conclusion, patients with only WT virus have the highest probability of achieving a combined HBeAg and HBV DNA response and HBsAg loss, whereas patients with PC or BCP mutants are predisposed to persistent viral replication after HBeAg clearance. This limits the use of HBeAg clearance alone when assessing therapy efficacy of PEG-IFN, and potentially other treatment options as well. Patients with WT virus appear to be the most suitable candidates for PEG-IFN therapy, irrespective of HBV genotype.

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