APJAI
Asian Pacific Journal of
Allergy and Immunology

ORIGINAL ARTICLE

Genetic variation in *STAT4* is associated with treatment response to pegylated interferon in patients with chronic hepatitis B

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Abstract

Background: Signaling pathways in the *STAT4* gene play an essential role in interferon-mediated antiviral effects.

Objective: This study was aimed at investigating the role of rs7574865, a single nucleotide polymorphism (SNP) in *STAT4*, in patients with chronic hepatitis B (CHB) treated with pegylated interferon (PEG-IFN).

Methods: A total 261 Thai patients (115 HBeAg-positive and 146 HBeAg-negative CHB) treated with 48-week PEG-IFN were recruited. Virological response (VR) at 48 weeks post treatment was defined as HBeAg seroconversion plus HBV DNA < 2,000 IU/mL for HBeAg-positive CHB and HBV DNA < 2,000 IU/mL for HBeAg-negative CHB. The SNP was analyzed by TaqMan PCR assay.

Results: The distribution of GG, GT and TT genotypes of rs7574865 was 41.8%, 42.9% and 15.3%, respectively. There was no different in its distribution according to HBeAg status. Overall, patients with TT genotype, compared with non-TT genotype, achieved higher VR (64.3% vs. 30.5%; $P < 0.001$) and HBsAg clearance (23.8% vs. 5.0%; $P < 0.001$). There was the same trend in the HBeAg-positive group (VR, 52.4% vs. 30.9%; $P = 0.077$; HBsAg clearance, 23.8% vs. 6.4%; $P = 0.028$) and in the HBeAg-negative group (VR, 68.4% vs. 32.3%; $P = 0.004$; HBsAg clearance, 21.1% vs. 4.7%; $P = 0.026$). Multiple regression analysis demonstrated that low baseline HBsAg level and TT genotype were factors independently associated with VR and HBsAg clearance.

Conclusions: Our data support that SNP rs7574865 is associated with response to PEG-IFN therapy in Thai patients with CHB, regardless of baseline HBeAg status. Thus, the determination of this SNP could maximize cost-effectiveness of PEG-IFN in patients with CHB.

Key words: Chronic hepatitis B, pegylated interferon, *STAT4*, single nucleotide polymorphism, virological response

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Introduction

Worldwide, hepatitis B virus (HBV) is a major etiological factor for the development of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC).[1] Pegylated interferon alfa (PEG-IFN), acting through its dual immunomodulatory and antiviral effects, is an approved agent for treatment of chronic hepatitis B (CHB).[2] It has been shown that long-term therapeutic effect of PEG-IFN is sustained and responders have a reduced risk of cirrhosis and HCC.[3] However, the overall response rates to PEG-IFN in HBeAg-positive and HBeAg-negative CHB are limited with approximately 30-40% and 20-30%, respectively.[2] Moreover, PEG-IFN treatment has some potential side effects and its efficacy varies among individuals. Thus, in clinical practice it is essential to identify patients with a high likelihood of response prior to initiate PEG-IFN therapy.

The therapeutic effectiveness of PEG-IFN in patients with CHB is associated with dynamic interaction of viral and host factor.[4] Regarding host genetic variations, a recent genome-wide association study (GWAS) demonstrated that a single nucleotide polymorphism (SNP), rs7574865 located in the third intron of the *signal transducers and activators of transcription 4* (*STAT4*) gene, was linked to an increased risk of HBV-associated HCC in Chinese populations.[5] Indeed, *STAT4* is a member of the STAT protein family that could be activated by several cytokines including IFN-alfa in response to viral infections.[6] Subsequent studies also showed that the SNP was related to increased risk of advanced fibrosis or cirrhosis in Chinese and Caucasians patients with CHB.[7,8] Recently, it was reported that patients with the minor T allele of rs7574865 was more likely to achieve spontaneous HBsAg clearance than those harboring the major G allele.[9,10] Interestingly, a recent report also showed that rs7574865 represented one of the most important factors associated with sustained response in Chinese patients with HBeAg-positive CHB receiving IFN or PEG-IFN therapy.[11] Despite these observations, the association of the SNP with PEG-IFN response in other ethnical populations or in patients with HBeAg-negative CHB remains to be confirmed. Thus, this study was aimed at investigating the predictive role of this SNP in Thai patients with HBeAg-positive and HBeAg -negative CHB receiving PEG-IFN therapy.

Materials and methods

Patients

Thai patients with HBeAg-positive or HBeAg-negative CHB, who were treated and completed a full course of PEG-IFN therapy between January 2010 and May 2015 at the King Chulalongkorn Memorial Hospital, Bangkok, Thailand were recruited in this study. These patients were treated with PEG-IFN-alfa2a (180 μg/week) or PEG-IFN-alfa2b (1.5 μg/kg body weight/week) for 48 weeks and followed up for at least 48 weeks after therapy. Patients who had previously received NA were eligible, but not within 6 months prior to PEG-IFN therapy. All these patients had serum HBsAg positivity for at least 6 months before therapy, with elevated serum alanine aminotransferase (ALT) and HBV DNA levels. Individuals co-infected with hepatitis C virus (HCV) and/or human immunodeficiency virus (HIV) were excluded. Virological response (VR) for HBeAg-positive CHB was defined as HBeAg seroconversion plus HBV DNA level < 2,000 IU/mL at 48 weeks post treatment. For the HBeAg-negative CHB group, VR was defined as HBV DNA level < 2,000 IU/mL at 48 weeks post treatment.[12]

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. Written informed consents were obtained from patients and the study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University (IRB No. 016/61).

Serological and virological assays

Qualitative measurements of HBsAg, HBeAg, anti-HBe and anti-HBs were tested by commercially available enzyme-linked immunosorbent assays (Abbott Laboratories, Chicago, IL). HBsAg quantification were performed by Elecsys HBsAg II Quant reagent kits (Roche Diagnostics, Indianapolis, IN). Serum HBV DNA quantification were performed by Abbott RealTime HBV assay (Abbott Laboratories, Chicago, IL). HBV genotypes were determined by direct sequencing, as previously described.[13]

Genotyping of rs7574865

Genomic DNA was isolated from 100 μL of buffy coat samples using phenol-chloroform extraction method. The SNP rs7574865 of *STAT4* was determined by a commercial TaqMan SNP genotyping assay (Applied Biosystems, CA, USA; Part number: C__29882391_10), as described previously.[14] The polymerase chain reaction (PCR) assay was performed according to the manufacturer's instructions. Fluorescent signals (FAM and VIC) were acquired at the end of each cycle. Allelic discrimination plot was analyzed using Applied Biosystems QuantStudio Real-Time PCR System Software (Applied Biosystems, CA, USA).

Liver stiffness measurement

Liver stiffness (LS) measurement was performed prior to PEG-IFN therapy using transient elastography (FibroScan, Echosens, Paris, France). Results were recorded in kilopascals (kPa) as the median value of all measurements. The procedure was based on at least 10 validated measurements: the success rate was over 60% and the interquartile range was less than 30%.[15]

Statistical Analysis

Statistical analysis was performed using SPSS version 22 software (SPSS, Chicago, IL, USA). The Mann-Whitney U test or Student's test were used to compare continuous variables, and the χ^2 test were used to compare categorical variables. Logistic regression was used to assess odd ratios of factors associated with VR and HBsAg clearance. *P*-values < 0.05 were considered statistically significant.

Results

Patient Characteristics

Of the 302 consecutive patients with completed a full course of treatment and followed up, 261 patients had complete clinical data and available blood samples for genomic DNA isolation and thus were included in this study. Among these patients, there were 115 and 146 patients with HBeAg-positive and HBeAg-negative CHB, respectively. **Table 1** summarizes demographic and baseline clinical characteristics of the patients in relation to HBeAg status. Briefly, the HBeAg-positive CHB group had a significantly lower mean age, but a higher proportion of male gender compared with the HBeAg-negative CHB group. In addition, the HBeAg-positive CHB group had higher baseline levels of ALT, $\log_{10}$ HBV DNA and $\log_{10}$ HBsAg compared with the HBeAg-negative CHB group. However, no significant difference between groups was observed regarding HBV genotype distribution and baseline LS measurement. Among the HBeAg-positive CHB group, VR and HBsAg clearance were achieved in 40 (34.8%) and 11 (9.6%) patients, respectively. Among the HBeAg-negative CHB group, the corresponding figures were 54 (37.0%) and 10 (6.8%), respectively.

Table 1. Baseline characteristics and treatment response in relation to HBeAg status

Characteristics	HBeAg-positive CHB (n = 115)	HBeAg-negative CHB (n = 146)	*P* value
Age, year	35.1 ± 8.8	41.4 ± 9.6	< 0.001*
Sex, male	79 (68.7%)	78 (53.4%)	0.015*
ALT (U/L)	114.1 ± 60.1	74.9 ± 35.7	< 0.001*
Log_{10} HBV DNA (IU/ml)	7.2 ± 0.9	5.4 ± 0.9	< 0.001*
Log_{10} HBsAg (IU/ml)	4.0 ± 0.6	3.4 ± 0.5	< 0.001*
HBV genotypes			0.608
B	17 (14.8%)	28(19.2%)	
C	94 (81.7%)	112 (76.7%)	
Missing	4 (3.5%)	6 (4.1%)	
Liver stiffness (kPa)	7.6 ± 3.3	8.0 ± 2.4	0.369
Virological response	40 (34.8%)	54 (37.0%)	0.795
HBsAg clearance	11 (9.6%)	10 (6.8%)	0.494
SNP rs7574865			0.251
GG	42 (36.5%)	67 (45.9%)	
GT	52 (45.2%)	60 (41.1%)	
TT	21 (18.3%)	19 (13.0%)	

ALT, alanine aminotransferase; HBsAg, Hepatitis B surface antigen; SNP, Single nucleotide polymorphism; Data described as means ± SD or n (%), * < 0.05

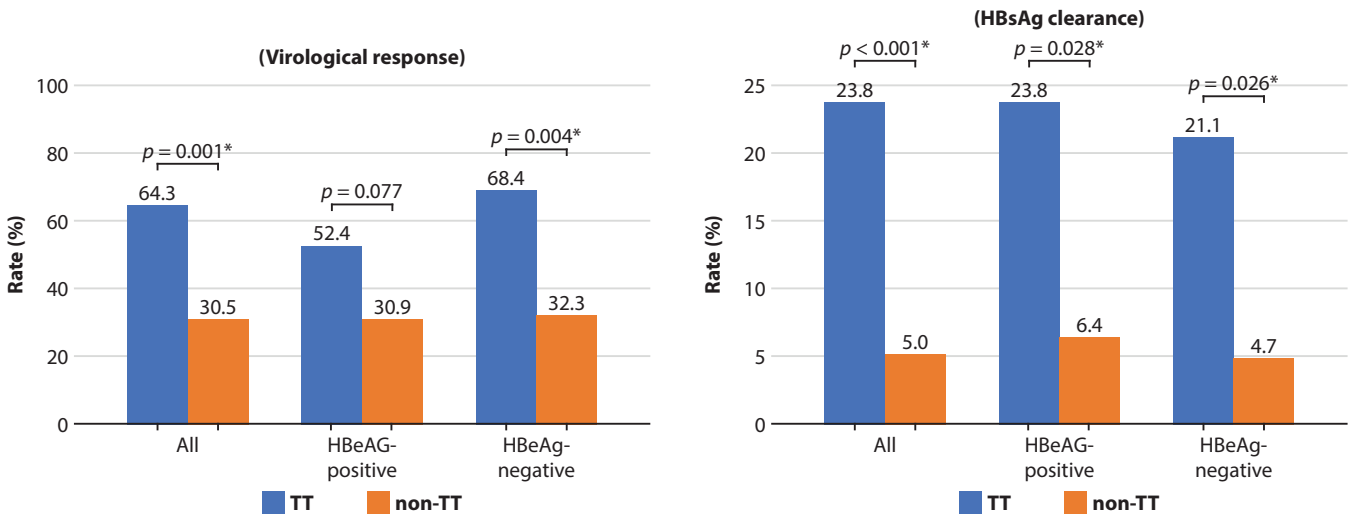

Figure 1. Treatment response in relation to rs7574865 genotypes

The frequency of rs7574865 genotypes and treatment response

The distribution of GG, GT and TT genotypes of rs7574865 in this cohort was 41.8%, 42.9% and 15.3%, respectively. The distribution of the corresponding genotypes in the HBeAg -positive CHB group were 42 (36.5%), 52 (45.2%) and 21 (18.3%), respectively, and in the HBeAg-negative CHB group were 67 (45.9%), 60 (41.1%) and 19 (13.0%), respectively, which was not significantly different between these two groups (P = 0.251).

Overall, patients with TT genotype, compared with non-TT genotype, achieved higher VR (64.3% vs. 30.5%; P < 0.001) and HBsAg clearance (23.8% vs. 5.0%; P < 0.001). There was the same trend in the HBeAg-positive group (VR, 52.4% vs. 30.9%; P = 0.077; HBsAg clearance, 23.8% vs. 6.4%; P = 0.028) and in the HBeAg-negative group (VR, 68.4% vs. 32.3%; P = 0.004; HBsAg clearance, 21.1% vs. 4.7%; P = 0.026) (**Figure 1**).

Serum HBsAg kinetics in associated with rs7574865 genotypes

Serum HBsAg kinetics in associated with rs7574865 genotypes were further investigated. Among HBeAg-positive CHB, patients with TT genotype showed a higher decline of serum HBsAg levels at the end of follow-up compared with those

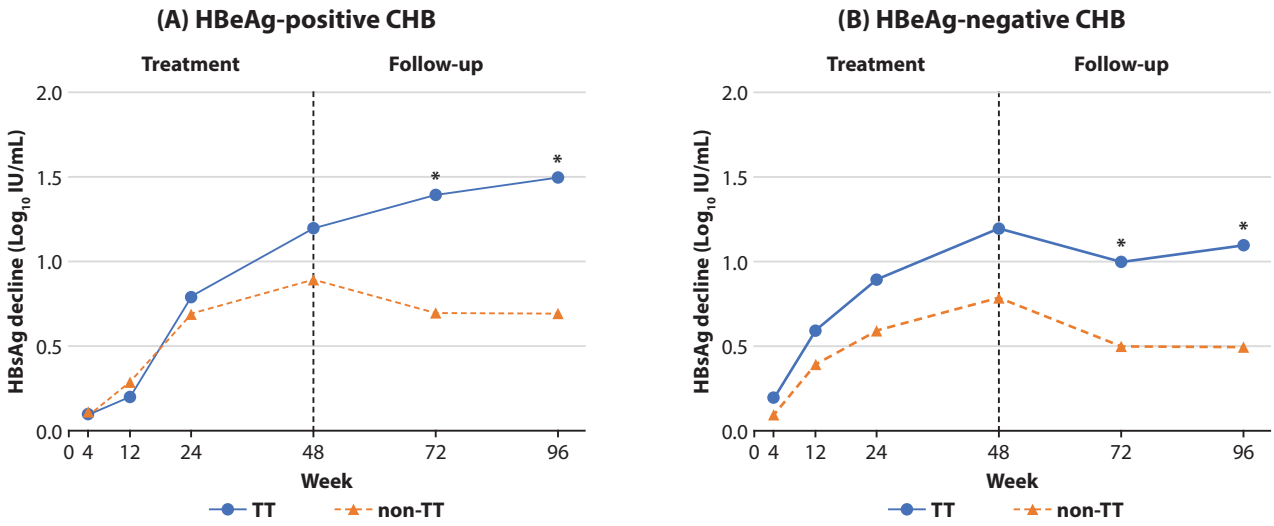

Figure 2. Serum HBsAg decline from baseline during and after therapy in relation to rs7574865 genotypes
*P < 0.05

Table 2. Logistic regression analysis of pretreatment factors to predict treatment response
(A) Virological response

Factors	Category	Virological response (week 96)			
		Univariate analysis		Multivariate analysis	
		Odd ratio (95%CI)	*P*	Odd ratio (95%CI)	*P*
Age (year)	≥ 40 vs. < 40	0.71 (0.42-1.19)	0.189		
Sex	Male vs. Female	1.19 (0.71-2.00)	0.503		
ALT (U/L)	≥ 100 vs. < 100	1.22 (0.70-2.15)	0.482		
Log_{10} HBV DNA (IU/mL)	< 7.0 vs. ≥ 7.0	1.36 (0.78-2.36)	0.282		
Log_{10} HBsAg (IU/mL)	< 3.5 vs. ≥ 3.5	2.82 (1.67-4.75)	< 0.001*	2.96 (1.73-5.02)	< 0.001*
HBV genotypes	B vs. C	1.02 (0.52-1.99)	0.963		
Liver stiffness (kPa)	≥ 8.0 vs. < 8.0	0.86 (0.49-1.51)	0.589		
SNP rs7574865	TT vs. Non-TT	1.80 (1.27-2.54)	0.001*	1.87 (1.30-2.68)	0.001*

(B) HBsAg clearance

Factors	Category	HBsAg clearance (week 96)			
		Univariate analysis		Multivariate analysis	
		Odd ratio (95%CI)	*P*	Odd ratio (95%CI)	*P*
Age (year)	≥ 40 vs. < 40	0.96 (0.39-2.37)	0.937		
Sex	Male vs. Female	1.15 (0.46-2.82)	0.769		
ALT (U/L)	≥ 100 vs. < 100	1.08 (0.40-2.89)	0.883		
Log_{10} HBV DNA (IU/mL)	< 7.0 vs. ≥ 7.0	1.68 (0.68-4.17)	0.261		
Log_{10} HBsAg (IU/mL)	< 3.5 vs. ≥ 3.5	2.71 (1.08-6.79)	0.034*	2.85(1.10-7.33)	0.030*
HBV genotypes	B vs. C	0.86 (0.27-2.72)	0.801		
Liver stiffness (kPa)	≥ 8.0 vs. < 8.0	0.65 (0.23-1.85)	0.420		
SNP rs7574865	TT vs. Non-TT	2.25 (1.40-3.60)	0.001*	2.30 (1.42-3.72)	0.001*

with non-TT genotype: baseline (4.0 ± 0.7 vs. 3.9 ± 0.6 $\log_{10}$ IU/mL, P = 0.924), week 4 (0.1 ± 0.3 vs. 0.1 ± 0.3 $\log_{10}$ IU/mL, P = 0.929), week 12 (0.2 ± 0.3 vs. 0.3 ± 0.7 $\log_{10}$ IU/mL, P = 0.257), week 24 (0.8 ± 1.0 vs. 0.7 ± 1.0 $\log_{10}$ IU/mL, P = 0.795), week 48 (1.2 ± 1.4 vs. 0.9 ± 1.1 $\log_{10}$ IU/mL, P = 0.253), week 72 (1.4 ± 1.5 vs. 0.7 ± 1.1 $\log_{10}$ IU/mL, P = 0.018), and week 96 (1.5 ± 1.5 vs. 0.7 ± 1.1 $\log_{10}$ IU/mL, P = 0.013) (**Figure 2A**).

Likewise, decline HBsAg levels at the end of follow-up were higher in patients with HBeAg-negative CHB harboring TT genotype than those with non-TT genotype: baseline (3.4 ± 0.5 vs. 3.4 ± 0.5 $\log_{10}$ IU/mL, P = 0.400), week 4 (0.2 ± 0.3 vs. 0.1 ± 0.2 $\log_{10}$ IU/mL, P = 0.301), week 12 (0.6 ± 0.8 vs. 0.4 ± 0.6 $\log_{10}$ IU/mL, P = 0.114), week 24 (0.9 ± 0.9 vs. 0.6 ± 0.8 $\log_{10}$ IU/mL, P = 0.177), week 48 (1.2 ± 1.2 vs. 0.8 ± 0.9 $\log_{10}$ IU/mL, P = 0.139), week 72 (1.0± 1.0 vs. 0.5 ± 0.8 $\log_{10}$ IU/mL, P = 0.028), and week 96 (1.1 ± 1.0 vs. 0.5 ± 0.8 $\log_{10}$ IU/mL, P = 0.008) (**Figure 2B**).

Factors associated with treatment response

To identify factors associated with VR and HBsAg clearance, baseline characteristics of patients were analyzed by logistic regression analyses. Potential predictors of virological response included sex, age, ALT level, HBV DNA level, HBsAg level, LS value and SNP rs7574865. As shown in **Table 2**, low baseline HBsAg level and SNP rs7574865 were factors independent associated with VR and HBsAg clearance.

If pretreatment HBsAg and SNP rs7574865 were combined together, patients carried TT genotype with baseline HBsAg < 3.5 $\log_{10}$ IU/mL achieved VR and HBsAg clearance of 81.3% (13/16) and 31.3% (5/16), respectively. Conversely, patients carried non-TT genotype with high baseline HBsAg ≥ 3.5 $\log_{10}$ IU/mL achieved VR and HBsAg clearance of 23.1% (31/103) and 3% (4/130), respectively.

Discussion

It has been well recognized that both viral- and host-related factors influence the natural history and treatment outcome of patients with HBV or HCV infection.[4] In the context of host genetic variation, a previous landmark GWAS identified SNPs in the *interferon lambda-3* (*IFNL3*) gene on chromosome 19 (19q13.13) associated with clearance and response to combined PEG-IFN and ribavirin therapy in patients with HCV infection.[16,17] Regarding HBV infection, a GWAS revealed that SNPs in the *human leucocyte antigen* (*HLA*) class II genes (*HLA-DP*) were related to spontaneous HBV clearance among Asian populations. Subsequent studies also validated that *HLA-DP* polymorphisms were association with response to PEG-IFN therapy.[18,19] More recently, a GWAS demonstrated that SNP rs7574865 in the *STAT4* gene was linked to an increased risk of developing HCC in Chinese individuals with CHB.[5] In addition, another Chinese cohort showed that rs7574865 TT genotype predicted HBeAg seroconversion in patients with HBeAg-positive CHB receiving IFN-based therapy.[11]

In this report, we investigated the effect of rs7574865 on treatment outcome to PEG-IFN in Thai population. Our study, recruited subjects with both HBeAg-positive and HBeAg-negative CHB, clearly showed that the frequency of rs7574865 TT genotype was significantly associated with favorable outcome to PEG-IFN. Specifically, subjects harboring TT and non-TT genotypes had the probabilities in achieving VR of approximately 65% and 30%, respectively. Moreover, the corresponding figures for attaining HBsAg clearance were 24% and 5%, respectively. Indeed, HBsAg clearance or 'functional cure' represents an ultimate goal of antiviral therapy, which is associated with improved clinical consequences, such as reduced incidence of cirrhosis and HCC. Together, these results might indicate that rs7574865 TT genotype was linked to PEG-IFN responsiveness in Thai individuals and the determination of this SNP might improve cost-effectiveness of the therapy. Of note, a recent study also indicated that rs7574865 were associated with response to tumor necrosis factor (TNF) inhibitors in patients with rheumatoid arthritis.[20]

STAT4 is a vital transcription factor for T helper[1] cell activation and differentiation that could be induced by several cytokines in response to viral infections to regulate inflammation and anti-viral activities.[6] Given that *STAT4* is a crucial signaling of the Janus kinase (JAK)-STAT pathway, through which IFN-alfa is modulated, variations in the *STAT4* gene might represent a good genetic predictor for immune-modulatory agents such as IFN-based therapy. As rs7574865 is located in the third intron of the *STAT4* gene, its potential effects might be associated with the alteration of *STAT4* expression. For instance, a recent study demonstrated that the presence of the minor T allele of rs7574865 enhanced *STAT4* mRNA transcription and protein expression in blood samples of healthy controls and patients with autoimmune disorders.[14,21] In systemic lupus erythematosus (SLE), it was recently shown that T allele of rs7574865 was associated with increased IL-12-induced IFN-gamma production in T cells.[22] In HBV-related HCC, a quantitative RT-PCR assay confirmed that the expression levels of *STAT4* mRNA were significantly lower in both tumor and adjacent tissues of patients harboring the risk G allele when compared to individuals with the T allele.[5] Another recent study in patents with CHB also showed that the G allele was associated with lower *STAT4* mRNA expression in liver and PBMCs, impaired *STAT4* phosphorylation and a reduction in IFN-gamma secretion following *ex vivo* stimulation.[8] Together, these results indicate that patients with CHB carrying the TT genotype are more likely to enhance *STAT4* expression, which might result in a higher likelihood of responsiveness to PEG-IFN therapy.

Currently, baseline predictors of response to PEG-IFN therapy in patients with CHB are not well defined. In this report, the response rates of patients with CHB were not influenced by age, gender, baseline ALT, HBV DNA level and HBV genotype. Instead, rs7574865 and pretreatment HBsAg level were selected as independent predictors of VR and HBsAg clearance in multivariate analysis. The finding that low baseline HBsAg level was predictive of response was in line with previous data in patients with HBeAg-positive and HBeAg-negative CHB treated with IFN-based therapy.[23-25] In fact, low serum HBsAg quantification might represent strong immune control,[26] and this may in part explain a favorable treatment outcome including VR and HBsAg clearance. Interestingly, the response rates were particularly high among subgroups of patients carrying favorable TT genotype, who also had low baseline HBsAg quantification. For example, patients harboring TT genotype with baseline HBsAg < 3.5 $\log_{10}$ IU/mL had a high chance of achieving VR and HBsAg clearance of approximately 80% and 30%,

respectively. Together, such findings provide insight into the virus–host interactions and indicate that combined use of these markers could further improve predictive value to some extent.

Although this is the first study that examines the association between rs7574865 genotypes and PEG-IFN responsiveness in patients with both HBeAg-positive and HBeAg-negative CHB, our report had some limitations as being a retrospective study with a relative small sample size of patients achieving HBsAg clearance. Nonetheless, our data clearly demonstrated that the SNP was an independent predictor of treatment outcome to PEG-IFN. Thus, the determination of this polymorphism, together with HBsAg quantification, is useful for identification of patients with high probability of response and may help to individualize decision-making before initiating PEG-IFN therapy. Further prospective large-scale analyses are, however, needed to confirm these observations.

Acknowledgements

This study was funded by The Thailand Research Fund (TRF) Senior Research Scholar (RTA5980008), the Grant for Chula Research Scholar (CU-GRS-60-06-30-03), the 100th Anniversary Chulalongkorn University for Doctoral Scholarship, the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and the Overseas Research Experience Scholarship for Graduate Students (for U.L.). The study was also supported by Center of Excellence in Hepatitis and Liver Cancer, the Research Chair Grant NSTDA (P-15-50004), Center of Excellence in Clinical Virology (GLE 58-014-30-004, RES560530093) Chulalongkorn University and Chulalongkorn Hospital.

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