Host genetic factors in predicting response status in chronic hepatitis B patients discontinuing nucleos(t)ide analogs

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Abstract

Background/Aims: The optimal duration of nucleos(t)ide analogs (NAs) therapy in chronic hepatitis B (CHB) patients remains unsatisfactory. Previous studies have confirmed the important role of host genetic factors in determining the outcome of HBV infection. This study tries to determine the role of host genetic factors in predicting response status in CHB patients discontinuing NAs according to stringent cessation criteria.

Patients and Methods: Participating patients came from a prospective NAs- discontinuation cohort since June 1999. Six single-nucleotide polymorphisms (SNPs) were selected according to previous report. SNaPshot assay was used for DNA SNPs analyses.

Results: Seventy-six CHB patients were enrolled in our study, of which 61 patients were HBeAg-positive and 15 patients were HBeAg-negative. rs1883832 in the Kozak sequence of CD40 displayed an AUROC of 0.778 in predicting response status in CHB patients with HBeAg seroconversion and a genotype of CT was associated with sustained response in this subpopulation. The diagnostic performance of combinative index (rs1883832, age, and HBsAg at discontinuation) seemed to be better than that of rs1883832, but no statistical difference was observed. rs1883832 was also evaluated as an independent factor for response status by multivariate logistic regression. For HBeAg-negative CHB patients, rs9277535 at HLA-DP presents a Spearman correlation coefficient of 0.582 ($P = 0.023$) with virological relapse after discontinuation of NAs.

Conclusions: rs1883832 serves as a valuable predictive factor for CHB patients with HBeAg seroconversion. rs9277535 at HLA-DP might also be a valuable predictive factor for CHB patients with HBeAg-negative, however, further verifications are recommended due to study limitations.

Keywords: Chronic hepatitis B, discontinuation, hepatitis B e antigen, nucleos(t)ide analogs, single-nucleotide polymorphism

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INTRODUCTION

Chronic hepatitis B (CHB) affects about 240 million people worldwide and is likely to cause liver fibrosis, which may lead to architectural distortion of the liver.[1]

Further progression of the disease may lead to cirrhosis, hepatic decompensation, and hepatocellular carcinoma. The introduction of nucleos(t)ide analogs (NAs) has significantly improved the prognosis of CHB;[1] however, discontinuation of NAs therapy still remains a conundrum

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in clinical practice. Although current guidelines have paid close attention to the optimal duration of NAs therapy, the "unknown" or "indefinite" treatment period is less than satisfactory.[1–3]

Several studies have been conducted to explore predictors for response status after discontinuation of NAs. Hepatitis B surface antigen (HBsAg) seroclearance is proved to be a favorable clinical outcome[4] and has been recommended as an optimal endpoint for discontinuation.[1–3] However, the scarce occurrence of HBsAg loss/conversion makes it unrealistic for most CHB patients.[5,6] The duration of consolidation therapy relates to the relapse rates in Hepatitis B e antigen (HBeAg)-positive CHB patients who have achieved HBeAg seroconversion.[2,7,8] However, the relapse rates remain high in HBeAg-negative CHB patients even when stringent cessation criteria are applied.[2,9] Our previous studies also revealed age as a predictive factor for relapse after discontinuation of NAs,[8,9] though a cut-off value of <30 years (HBeAg-positive CHB patients) or <20 years (HBeAg-negative CHB patients) for lower relapse rate may not be a good news for older CHB patients.

The progression of CHB depends on interaction between human body and hepatitis B virus (HBV). Previous studies have confirmed the important role of host genetic factors in determining the outcome of HBV infection.[10] Several single-nucleotide polymorphisms (SNPs) involving progression of CHB have been revealed by genome-wide association studies (GWASs) recently, most of which locate in human leukocyte antigen (HLA) regions.[11–16] A new locus locating at 20q13.1 (rs1883832 in the Kozak sequence of CD40) was also found to be associated with CHB susceptibility in Chinese population by Jiang *et al.* in 2015.[11] However, there are few studies focusing on the correlation between host genetic factors and response status after discontinuation of NAs therapy. Based on our prospective NAs- discontinuation cohort and the follow-up for more than 10 years, we conducted this study to define the role of host genetic factors in predicting response status in CHB patients discontinuing NAs by applying stringent cessation criteria.

PATIENTS AND METHODS

Patients
Our study participants came from prospective NAs- discontinuation cohort since June 1999. Informed consents were obtained from all patients. Our study protocol followed guidelines of the 1975 Helsinki Declaration and standards of the Ethical Committee of our hospital.

We followed our previous studies in terms of indications and cessation criteria.[8,9] In summary, the presence of serum HBsAg should be observed for more than 6 months before antiviral therapy is prescribed to the patients. The indications of therapy were alanine aminotransferase (ALT) levels no less than two times the upper limit of normal (ULN, 40 IU/L) and serum HBV DNA levels no less than 10^5 copies/mL (HBeAg-positive CHB patients) or 10^4 copies/mL (HBeAg-negative CHB patients). The cessation criteria were defined as follows: (1) HBeAg-positive patients: at least 6 months' additional consolidation treatment after achieving HBeAg seroconversion with undetectable HBV DNA and normal ALT plus a total therapy duration more than 12 months. Some CHB patients with HBeAg loss also underwent discontinuation of NAs prudently with more than 18 months' total therapy duration and at least 6 months' consolidation treatment before December 2013. (2) HBeAg-negative patients: at least 18 months' additional consolidation treatment after achieving undetectable HBV DNA and ALT plus at least 24 months' total treatment duration. Virological relapse was defined as the reappearance of serum HBV DNA ($>10^4$ copies/mL) by PCR assay.

Patients with virological relapse accepted various salvage treatments according to their previous antiviral agents and the severity of liver disease.

Single-nucleotide polymorphisms
We performed a systematic literature search in PubMed, EMBASE, and Web of Science for selection of SNPs. The search terms used was "HBV or hepatitis B virus", "SNP or Single Nucleotide Polymorphism". Six SNPs were selected according to previous report[11–16] and were confirmed from SNP database in NCBI (www.ncbi.nlm.nih.gov/SNP): rs9277535 at HLA-DP, rs7453920 at HLA-DQ, rs2856718 at HLA-DQ, rs12614 in complement factor B, rs422951 in NOTCH4, rs1883832 in the Kozak sequence of CD40. DNA was extracted from the peripheral blood mononuclear cells of each patient (Generay Biotech, Shanghai). SNaPshot assay (Applied Biosystems, Foster City, CA) was used for DNA SNPs analyses. All primer and probe sequences are available upon request.

Statistical analysis
Continuous variables were described as either mean ± standard deviation (SD) or median (interquartile range (IQR)). These variables were compared with *t* test or Mann–Whitney U test as appropriate. Categorical variables were compared with χ^2 test. Fisher's exact test was also used when necessary. Spearman correlation coefficient was used to evaluate the correlation between

SNPs and response status. Multivariate logistic regression was performed to assess SNPs as independent factors for response status. Comparisons of areas under the receiver operating characteristic curve (AUROCs) were performed according to Hanley *et al*.[17] A $P < 0.05$ was considered as statistically significant. All statistical analyses were conducted using software SPSS 17.0 (SPSS, Chicago, IL) and MedCalc 12.7.0.0 (MedCalc Software bvba, Ostend, Belgium). Minor Allele Frequency (MAF) and the Hardy–Weinberg equilibrium of SNPs were tested using SNPStats (http://bioinfo.iconcologia.net/SNPstats).

RESULTS

Study patients

Seventy-six CHB patients were included in our study, of which 61 patients were HBeAg-positive (34 HBeAg seroconversion and 27 HBeAg loss/no seroconversion to anti-HBe) and 15 patients were HBeAg-negative. Seven patients achieved HBsAg loss/conversion by the time of discontinuation. Another 10 patients were found to achieve HBsAg loss/conversion later on during follow-up. Most patients were males (73.7%). The overall mean age was 30.3 ± 11.4 years. The initial treatment strategies were lamivudine in 42 patients, adefovir in 20 patients, telbivudine in 9 patients, and entecavir in 5 patients. The median treatment duration was 39 months (IQR 30–55 months) and the median follow-up period was 48 months (IQR 7–84 months).

Twenty-eight patients relapsed after a median of 39 months' follow-up, 7 patients in HBeAg seroconversion group, 12 patients in HBeAg loss group, and 9 patients in HBeAg-negative group. Patients with sustained response were found to be younger ($P = 0.006$) and more HBsAg loss/conversion ($P = 0.042$) than relapse patients. The characteristics at discontinuation and SNPs of the study patients are summarized in Table 1.

Correlation of SNPs and response status

Basic genetic characteristics and analysis of Hardy–Weinberg equilibrium of the selected 6 SNPs are shown in Table 2. We found no correlation between SNPs and response status in total population [Table 1]. Because of the different features between HBeAg-positive and HBeAg-negative patients, we re-evaluated the correlation between SNPs and response status in 3 subgroups (HBeAg seroconversion group, HBeAg loss group, and HBeAg-negative group) [Table 3].

In CHB patients with HBeAg seroconversion, we found that rs1883832 in the Kozak sequence of

Table 1: Characteristics at discontinuation and single-nucleotide polymorphisms of the study patients

	Patients with sustained response (*n*=48)	Patients with relapse (*n*=28)	*P* *
Age (years)	27.6±10.3	34.9±11.9	0.006
Male/Female	35/13	21/7	0.842
HBsAg loss/conversion at discontinuation	7 (14.6%)	0 (0.0%)	0.042
Treatment duration (months, median, IQR)	39 (29-51)	40 (31-61)	0.319
rs9277535			
A/G	23 (47.9%)	10 (35.7%)	0.358
G/G	16 (33.3%)	14 (50.0%)	
A/A	9 (18.8%)	4 (14.3%)	
rs7453920			
A/G	6 (12.5%)	1 (3.6%)	0.250
G/G	42 (87.5%)	27 (96.4%)	
rs2856718			
A/G	23 (47.9%)	17 (60.7%)	0.414
G/G	4 (8.3%)	3 (10.7%)	
A/A	21 (43.8%)	8 (28.6%)	
rs12614			
C/T	3 (6.3%)	2 (7.1%)	0.358
C/C	45 (93.8%)	25 (89.3%)	
C/G	0 (0.0%)	1 (3.6%)	
rs422951			
A/G	11 (22.9%)	6 (21.4%)	0.381
G/G	2 (4.2%)	0 (0.0%)	
A/A	35 (72.9%)	22 (78.6%)	
rs1883832			
C/T	26 (54.2%)	11 (39.3%)	0.259
C/C	14 (29.2%)	8 (28.6%)	
T/T	8 (16.7%)	9 (32.1%)	

**t test or Chi-square test. IQR, interquartile range*

CD40 was correlated with response status after NAs discontinuation ($P = 0.042$, χ^2 test). A Spearman correlation coefficient of 0.424 ($P = 0.013$) also revealed the same conclusion. The percentage of genotype CT at rs1883832 was higher in patients with sustained response than that in relapse patients (59.3% vs. 14.3%), meanwhile the percentage of genotype TT was higher among relapse patients (57.1% vs. 14.8%) [Table 4]. In multivariate logistic regression analysis involving rs1883832, age at discontinuation and HBsAg status at discontinuation (Forward LR method), only rs1883832 was considered as a predictor for response status (OR 3.932, 95%CI 1.212–12.759, $P = 0.023$) [Table 5].

For HBeAg-negative CHB patients, rs9277535 at HLA-DP showed a Spearman correlation coefficient of 0.582 ($P = 0.023$) with response status after discontinuation of NAs, although a P value of 0.059 for χ^2 test [Table 3] did not indicate a statistical significance.

Diagnostic performances of SNPs and routine index for response status in patients with HBeAg seroconversion

According to our results, we assessed the diagnostic performance of rs1883832, age at discontinuation, and

Table 2: Basic genetic characteristics of the SNPs selected in this study

SNP	Chr	Position	Alleles[#]	Gene	MAF	Hardy-Weinberg equilibrium (*P*)
rs9277535	6	33087084	A/G	HLA-DPB1	0.39	0.47
rs7453920	6	32762235	A/G	HLA-DQB2	0.05	1
rs2856718	6	32702478	G/A	HLA-DQA2	0.36	0.31
rs12614	6	31946402	T/C	CFB	0.03	1
rs422951	6	32220606	G/A	NOTCH4	0.14	0.62
rs1883832	20	46118343	T/C	CD40	0.47	0.82

[#]Minor allele/major allele

Table 3: Correlation of single-nucleotide polymorphisms and response status

SNPs	Genotype (*n*,%) & *P* (χ^2 test)		
	HBeAg seroconversion group	HBeAg loss group	HBeAg-negative group
rs9277535 (AG/GG/AA)	18 (52.9%)/10 (29.4%)/6 (17.6%) 0.956	10 (37.0%)/11 (40.7%)/6 (22.2%) 0.804	5 (33.3%)/9 (60.0%)/1 (6.7%) 0.059
rs7453920 (AG/GG/AA)	3 (8.8%)/31 (91.2%)/0 (0.0%) 1.000	4 (14.8%)/23 (85.2%)/0 (0.0%) 0.605	0 (0.0%)/15 (100.0%)/0 (0.0%) –
rs2856718 (AG/GG/AA)	18 (52.9%)/3 (8.8%)/13 (38.2%) 0.783	14 (51.9%)/2 (7.4%)/11 (40.7%) 0.169	8 (53.3%)/2 (13.3%)/5 (33.3%) 0.126
rs12614 (CC/CG/CT)	4 (11.8%)/30 (88.2%)/0 (0.0%) 0.180	1 (3.7%)/26 (96.3%)/0 (0.0%) 1.000	14 (93.3%)/1 (6.7%)/0 (0.0%) 0.600
rs422951 (AG/GG/AA)	11 (32.4%)/1 (2.9%)/22 (64.7%) 0.663	6 (22.2%)/0 (0.0%)/21 (77.8%) 1.000	0 (0.0%)/1 (6.7%)/14 (93.3%) 0.400
rs1883832 (CT/CC/TT)	17 (50.0%)/9 (26.5%)/8 (23.5%) 0.042	12 (44.4%)/9 (33.3%)/6 (22.2%) 0.710	8 (53.3%)/4 (26.7%)/3 (20.0%) 0.676

SNP: Single-nucleotide polymorphism; HBeAg: Hepatitis B e antigen

Table 4: Correlation of genotypes of rs1883832 and response status in patients with HBeAg seroconversion

Genotypes	Patients with relapse (*n*,%)	Patients with sustained response (*n*,%)	Total (*n*,%)	*P**
CT	1 (14.3%)	16 (59.3%)	17 (50.0%)	0.042
CC	2 (28.6%)	7 (25.9%)	9 (26.5%)	
TT	4 (57.1%)	4 (14.8%)	8 (23.5%)	
Total (*n*)	7 (100%)	27 (100%)	34 (100%)	

*Chi-square test. HBeAg, Hepatitis B e antigen. *n*, number of patients. Percentage (%) means the constituent ratio of genotype (CT, CC or TT) in patients with sustained response and in relapse patients, respectively.

Table 5: Factors related to response status by univariate and multivariate logistic regression analyses

	Univariate			Multivariate[&]		
	OR	95%CI	*P*	OR	95%CI	*P*
Age[#]	1.436	0.269–7.678	0.672			
rs1883832	3.932	1.212–12.759	0.023	3.932	1.212–12.759	0.023
HBsAg status	471180320.7	0.000–	0.999			

[#]>25 years vs. ≤25 years; [&]Forward LR method

Figure 1: Diagnostic performance of rs1883832, age at discontinuation, and HBsAg status at discontinuation for response status in patients with HBeAg seroconversion. Rs1883832 displayed a better performance than HBsAg at discontinuation (*P* = 0.031). However, there was no significant difference for the diagnostic performance between rs1883832 and age (*P* = 0.602). Likewise, no statistical difference was found between the performance of age and that of HBsAg (*P* = 0.169)

HBsAg status at discontinuation for response status in patients with HBeAg seroconversion [Table 6]. Rs1883832 displayed a better performance than HBsAg at discontinuation (*P* = 0.031); however, there was no significant difference for the diagnostic performance between rs1883832 and age (*P* = 0.602). Likewise, no statistical difference was found between the performance of age and that of HBsAg (*P* = 0.169) [Figure 1].

We also assessed the performance of the combination of the 3 index (rs1883832, age, and HBsAg at discontinuation), which gave an AUROC of 0.854 (95%CI 0.691–0.951). We consider that the performance of combinative index does not differentiate from that of rs1883832 or age at discontinuation statistically, though the former appears
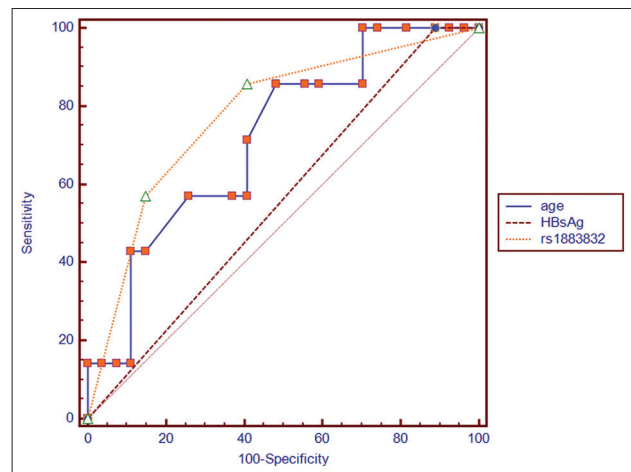
to be better (combination vs. rs1883832, *P* = 0.189; combination vs age, *P* = 0.107). The combinative index also displayed a better performance than HBsAg at discontinuation (*P* = 0.007) [Figure 2].

DISCUSSION

As far as we know, this is the first study to explore the role of host genetic factors in predicting response status in CHB patients discontinuing NAs. We found no correlation between SNPs and the response status in the overall population; however, rs1883832 in the Kozak sequence of CD40 displayed an AUROC of 0.778 in predicting response status in CHB patients with HBeAg seroconversion and

Table 6: Diagnostic performances of single-nucleotide polymorphisms and routine index for response status in patients with HBeAg seroconversion

Indicators	AUROC (95% CI)	Cut-off value	Sensitivity(%)	Specificity(%)	LR+	LR-
rs1883832	0.778 (0.603-0.902)	-	85.7 (42.1-99.6)	59.3 (38.8-77.6)	2.10	0.24
age	0.717 (0.537-0.857)	25 y	85.7 (42.1-99.6)	51.9 (31.9-71.3)	1.78	0.28
HBsAg	0.556 (0.376-0.725)	-	100.0 (59.0–100.0)	11.1 (2.4–29.2)	1.12	0.00

AUROC: Area under the receiver operating characteristic curve; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio
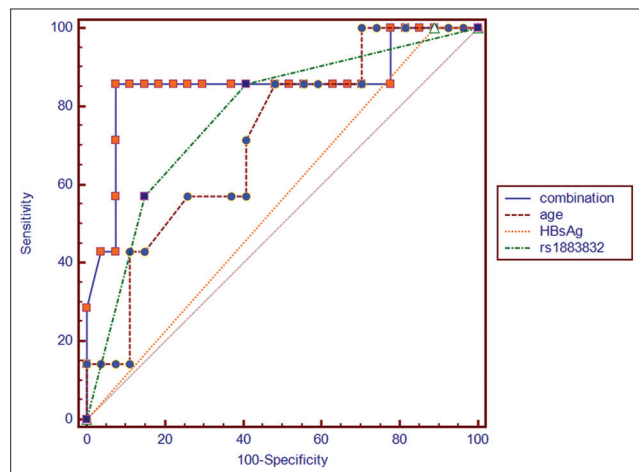

Figure 2: Comparison of diagnostic performance of combinative index (rs1883832, age at discontinuation, and HBsAg status at discontinuation) and 3 single indicators in predicting response status for patients with HBeAg seroconversion. Though with a better diagnostic performance than HBsAg at discontinuation (*P* = 0.007), the performance of combinative index does not differentiate from that of rs1883832 or age at discontinuation statistically (combination VS. rs1883832, *P* = 0.189; combination VS. age, *P* = 0.107)

a genotype of CT was associated with sustained response in this subpopulation. In addition, rs1883832 was also assessed as an independent factor for response status by multivariate logistic regression. Furthermore, rs9277535 at HLA-DP displayed an AUROC of 0.796 in predicting response status in HBeAg-negative CHB patients. A Spearman correlation coefficient of 0.582 (*P* = 0.023) may also approve the predictive value of rs9277535 for HBeAg-negative CHB patients.

The cumulative relapse rates and predictive factors in HBeAg-positive CHB patients and HBeAg-negative CHB patients were different.[2,3,8,9] For HBeAg-positive patients with HBeAg seroconversion, a relatively better response status may be achieved with the application of stringent discontinuation criterion.[8,9] Longer consolidation therapy after HBeAg seroconversion was preferred to obtain better response.[2,7,18] Age at discontinuation was also found as a predictor for response after cessation of NAs.[7,8,19] In the present study, age displayed an AUROC of 0.717 (sensitivity 85.7%, specificity 51.9%) in predicting response status in CHB patients with HBeAg seroconversion. The cut-off value of 25 years was much younger than a previous study (40 years),[19] which may be attributed to the relatively

younger mean age in our cohort (30.3 ± 11.4 years vs. 39.0 ± 10.3 years[19]).

CD40 is considered to play an important antiviral role in both humoral and cellular immunity.[11] A previous study revealed that the activation of CD40 was essential in rescuing HBV-specific CD8+ T cells from functional inhibition.[20] rs1883832 in the Kozak sequence was found to influence CD40 protein expression in B cells.[11,21] Our study also found that rs1883832 is a predictive factor for response status after discontinuation in CHB patients with HBeAg seroconversion and a genotype of CT is associated with sustained response, which has not been reported in any previous study to the best of our knowledge. The diagnostic performance of rs1883832 was similar to age at discontinuation, and both of them were better than HBsAg at discontinuation. We believe that the combination of rs1883832, age, and HBsAg at discontinuation could perform better than any single factor, and despite a better AUROC of 0.854, there were no significant differences. A larger sample group is required in future studies in order to achieve more reliable and comprehensive conclusions.

The risk of recurring viremia was high for HBeAg-positive patients failing to obtain HBeAg seroconversion.[22] In approaching this conclusion, our study included 27 HBeAg-positive patients with HBeAg loss, of which 12 patients relapsed after discontinuation of NAs. In this process, we did not find any SNP with predictive value for response status. Diagnostic tests of age (*P* = 0.294) and HBsAg at discontinuation (*P* = 0.106) delivered similar findings.

The cumulative relapse rates in HBeAg-negative CHB patients after discontinuation were high despite following stringent cessation criteria.[9,23] Our previous study showed that age was a predictive factor for sustained response with a cut-off value of 20 years.[9] Another study also found HBV-DNA ≤10⁵ IU/mL as an independent factor for sustained response.[23] Despite the above studies, HBsAg seroclearance was considered as the safest predictive factor for HBeAg-negative CHB patients.[2,3,24] However, the scarce occurrence and long time to HBsAg loss/ conversion have proven as obstacles for most patients. Our study included 15 CHB patients with HBeAg-negative,

of which no patient achieved HBsAg loss/conversion at cessation. Two patients achieved HBsAg loss/conversion after discontinuation of NAs during follow-up and 6 patients maintained sustained response with a follow-up time ranging from 26 to 150 months. Some other valuable predictors except for HBsAg seroclearance should be actively sought.

Class HLA-II molecules are expressed in antigen-presenting cells and play a critical role in the immune response of CD4^{+} T cells in participating both immune inflammatory response and autoimmune tolerance; therefore, HLA-DP molecules may be important in HBV elimination and progression of CHB.[16,25] rs9277535 at HLA-DPB1 may affect the process of antigen-presenting by encoding β-chain paralogs of HLA-DP.[16,25] Our study revealed that rs9277535 might be a valuable predictive factor for response status in HBeAg-negative CHB patients after discontinuation. We believe that a larger sample is required in future studies with the purpose of obtaining more reliable and comprehensive conclusions in this regard.

Despite the relatively small sample size, in view of the statistical significance of *P* value, especially for the receiver operating characteristic curve and Spearman correlation analysis, we feel confident that the size of the sample is large enough to service the purpose of this study, which makes our conclusions valid. We have no intention to reveal all valuable predictive factors. More of them are expected to be revealed by future studies. Besides, this research drew conclusions from multivariate logistic regression analysis in HBeAg-positive CHB patients though we think similar analysis in HBeAg-negative CHB patients might also be of great significance if larger sample size was included.

In conclusion, the present study revealed rs1883832 in the Kozak sequence of CD40 as a valuable predictive factor for CHB patients with HBeAg seroconversion and a genotype of CT was associated with sustained response. rs9277535 at HLA-DP might also be a valuable predictive factor for CHB patients with HBeAg-negative, however, further verifications are recommended due to study limitations.

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Conflicts of interest

There are no conflicts of interest.

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