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Development and validation of a new predictive model for the immune tolerance stage of chronic HBV infection based on the liver histopathological changes

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Abstract

Objective To identify clinical and viral indicators for the development of a new model to accurately differentiate the stages of chronic hepatitis B virus (HBV) infection based on histopathological changes in the liver.

Methods Clinical and liver pathology data from chronic hepatitis B (CHB) patients who underwent liver biopsy were retrospectively collected. The patients were allocated into test and validation groups. The area under the receiver operating characteristic (ROC) curve (AUC) was calculated to idneitfy the optimal diagnostic value for differentiating the stages of chronic HBV infection.

Results A total of 118 patients and 73 patients who met the diagnostic and inclusion criteria were selected as the test group and validation group, respectively. Multivariate analysis revealed that HBeAg was independently correlated with the IT and IC stages. The cutoff value of HBeAg used to quantitatively differentiate between IT and IC was 1335 S/CO. The AUC values were 0.921 (95% confidence interval (CI): 0.836–0.971) and 0.846 (95% CI: 0.726–0.967) in the test and validation groups, respectively. A new prediction model of the IT stage was established by using three indicators, namely, HBeAg, HBsAg and HBV DNA. The AUC values were 0.923 (95% CI: 0.864–0.982, $p < 0.001$) and 0.89 (95% CI: 0.787–0.994, $p < 0.01$) in the test and validation groups, respectively, when this prediction model was used. For the new model, CMA guidelines (2019 version), EASL guidelines (2017 version) and AASLD guidelines (2018 version), the error rates in the test group were 4.65%, 11.62%, 23.26%, and 46.51%, respectively, while the errors rates in the validation group were 20.0%, 25.0%, 40.0%, and 45.0%, respectively.

Conclusions High levels of HBeAg, rather than HBeAg positivity, may serve as a predictor of the IT stage. A predictive model for the immune tolerance stage was established by combining three indicators. Compared with the recommended standards from multiple current guidelines, the new prediction model has a significantly lower error rate.

Keywords Chronic HBV infection, Immune stage, Liver histopathology, Natural history

Introduction

According to a report from the World Health Organization, 20–30% of the approximately 240 million chronic hepatitis B virus (HBV) infections worldwide develop into cirrhosis and/or hepatocellular carcinoma (HCC) [1]. In the course of chronic HBV infection, the long-term interaction between the host and the HBV leads

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to repeated fluctuations in intrahepatic inflammation, the occurrence of liver fibrosis, and disease progression [2]. On the basis of changes in alanine aminotransferase (ALT) levels, HBV DNA, hepatitis B e-antigen (HBeAg), and liver pathological damage, the natural history of chronic HBV infection can be divided into four stages: immune tolerance (IT), immune clearance (IC), immune control (ICO), and immune reactivity (IR). These four stages have different virological, biochemical, and histological characteristics. This classification method plays an important role in guiding the diagnosis, treatment, and prognostic evaluation of chronic HBV infection [3, 4].

Previous studies have shown that the HBV-specific immune response determines the outcome of HBV infection [5]. Dysregulated or exhausted virus-specific T and B-cell functions persist during chronic HBV infection, but no distinct immune signature based on T and B cells is available for clinical subtyping [6]. Recently, Park et al. [7] reported the presence of HBV-specific T cells in patients with the IT stage. Mason et al. [8] reported the presence of hepatocyte clones within the liver tissue of patients in the IT stage. Many liver biopsy studies have revealed different degrees of inflammation and fibrosis in some patients with so-called"immune tolerance" [9, 10]. Some scholars have expressed different opinions on the IT stage of chronic HBV infection [11]. In view of these debates, in 2017, the European Association for the Study of the Liver (EASL) proposed a reclassification of the natural history of chronic HBV infection into five stages instead of the previous four stages [12]. However, no international consensus has been reached regarding this new classification [13, 14]. The guidelines issued by the EASL, American Association for the Study of Liver Diseases (AASLD), Asian Pacific Association for the Study of the Liver (APASL), and Liver Diseases and Infectious Diseases Society of the Chinese Medical Association (CMA) have recommended staging the natural history of chronic HBV infection. The main evaluation indicators used are consistent, such as ALT levels, HBV DNA, HBeAg, and hepatitis B surface antigen (HBsAg) quantification. However, there are still obvious differences in the definition level of each indicator. In particular, HBeAg positive and negative and not quantitative detection were used to differentiate the four stages [12, 15–17]. These differences led to inconsistencies with respect to the inclusion criteria in many basic and clinical studies and affected the evaluation of the diagnosis and prognosis of patients with chronic hepatitis B (CHB) [18]. Further unification needs to be explored. Therefore, on the basis of histopathological changes in the liver, the levels of clinical and viral indicators between the IT and IC stages or the ICO and IR stages were analyzed to provide more reasonable guidance for the staging and treatment of CHB. A new predictive model for the immune tolerance stage of chronic HBV infection was developed and validated herein.

Patients and methods

Patients

The clinical and pathological data of patients with CHB who underwent liver biopsies were collected for retrospective analysis as the test group at Taizhou Hospital of Zhejiang Province affiliated with Wenzhou Medical University from 2015 to 2022. The patients in the validation group were from Nanjing Drum Tower Hospital from 2017 to 2022. The diagnosis and staging of CHB were performed according to the Chinese Medical Association Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2019 edition) [15]. The exclusion criteria were as follows: ① patients with hepatitis A, C, D, or E virus infection, cytomegalovirus, Epstein–Barr virus infection, or human immunodeficiency virus infection; ② patients treated with interferon-α or nucleos(t)ide analogs previously; ③ patients with moderate to severe fatty liver, alcoholic liver, drug liver, autoimmune, or hereditary liver disease; ④ patients with liver cancer and cancer of other organs; and ⑤ patients with decompensated cirrhosis with severe heart, lung, or kidney disease or diabetes. CHB was staged on the basis of HBsAg, HBV DNA, HBeAg, ALT levels and liver pathology. The selected patients were categorized into the IT, IC, ICO, and IR stages. The criteria for the IT stages were as follows: HBsAg >4 log IU/ml, HBeAg positive, HBV DNA >7.3 log IU/ml, normal ALT levels, and liver pathology without obvious inflammation or fibrosis. The criteria for the IC stage were as follows: HBsAg positive, HBeAg positive, HBV DNA >4.3 log IU/ml, ALT levels continuously or repeatedly increased, and obvious inflammatory necrosis and/or fibrosis according to liver histology. The criteria for the ICO stage were as follows: HBsAg <3 log IU/ml, HBeAg negative, HBV DNA <3.3 log IU/ml, normal ALT levels, no or only mild inflammation and/or different degrees of fibrosis according to liver histology. The criteria for the IR stage were as follows: HBsAg positive, HBeAg negative, HBV DNA ≥3.3 IU/ml, ALT levels continuously or repeatedly increased and obvious inflammatory necrosis and/or fibrosis according to liver histology [15]. This study was approved by the Ethical Commission of Taizhou Hospital of Zhejiang Province and the Ethics Committee of Nanjing Drum Tower Hospital, Jiangsu Province. All patients signed an informed consent form. This study adhered to the Declaration of Helsinki.

Liver biopsy and pathological diagnosis

Liver samples were obtained using a 16-gauge Menghini needle that was guided by ultrasound. The length of the

biopsy specimen was longer than 15 mm. Hematoxylin and eosin (H&E) staining and Masson's trichrome staining were routinely performed. The inflammation grade and fibrosis stage of the liver biopsy samples were determined according to the Scheuer scoring system [15]. All biopsy samples were independently evaluated by two hepatopathologists. The degree of liver inflammatory necrosis was categorized as mild (G0–G1), moderate (G2–G3), or severe (G4). No obvious inflammation indicated a histological grade of G0–G1, and obvious inflammation indicated ≥G2. The degree of liver fibrosis was categorized as mild (F0–F1), moderate (F2–F3), or severe (F4). No obvious fibrosis was defined as a histological grade of F0–F1, and obvious fibrosis was defined as a grade ≥F2. A degree of hepatic steatosis ≥F2 was defined as moderate to severe fatty liver. The number of misclassified IT stage cases was calculated by subtracting the number of histologically confirmed'true'IT cases (based on liver biopsy) from the number of IT cases identified by different criteria (excluding pathological indicators). The error rate was calculated using the misclassified IT cases according to the AASLD criteria as the denominator. The following formula was added: Error rate (%) = [IT cases (by different criteria) − IT cases (histologically confirmed)] ÷ [IT cases (AASLD criteria) − IT cases (histologically confirmed)] × 100%.

Detection of serum markers and HBV DNA quantification

Serum HBsAg levels were quantified using an automated chemiluminescence immunoanalyzer (Alinity, Abbott Laboratories, USA). The kit was purchased from Abbott Laboratories. The range of detection was 0.05–250 IU/mL. If the sample concentration was greater than 250 IU/mL, the sample was diluted and retested. Semiquantitative detection of serum HBeAg and hepatitis B core antibody (HBcAb) (S/CO, where S represents the absorbance of the sample and CO represents the cutoff value) levels was also performed using an automated chemiluminescence immunoanalyzer (Alinity, Abbott Laboratories, USA). S/CO ≥1 is positive. S/CO <1 is negative. Serum HBV DNA was detected using a quantitative PCR instrument (ABI, USA). The kit was purchased from Shanghai Haoyuan Biological Technology Co., Ltd. The lower detection limit was 20 IU/mL.

Statistical analysis

All the statistical analyses were performed using SPSS 26.0 (IBM, NY, USA). Data with a normal distribution are expressed as the mean ± standard deviation. Student's *t* test was used to compare the IT versus IC stages and the ICO versus IR stages. Data that were not normally distributed are shown as medians (quartile intervals). The Mann–Whitney *U* test was used to test the IT and IC stages and the ICO and IR stages. For categorical variables, the number of cases (percentages) is indicated, and the chi-square test or Fisher's exact test was performed. The Hosmer–Lemeshow test was used to assess the goodness of fit of the model. The area under the receiver operating characteristic (ROC) curve (AUC) was used to evaluate the efficacy of the model during the IT, IC, ICO and IR stages, with a preset cutoff using the maximum Youden index. Differences between the ROCs were compared using the Deron test. A two-tailed p value <0.05 was considered statistically significant.

Results

Baseline data for the collected patients

The data of 118 patients in the test group, comprising 83 males and 35 females, who met the diagnostic and inclusion criteria were collected and used in this analysis. The mean age was 43 years, and 76 HBeAg-positive and 42 HBeAg-negative patients were included. The data of 73 patients in the validation cohort, comprising 44 males and 29 females, who met the diagnostic and inclusion criteria were also collected and used in this analysis. The mean age was 37 years, and 40 HBeAg-positive patients and 33 HBeAg-negative patients were included. The baseline clinical data and pathological changes in liver tissue are shown in Table 1.

Comparative analysis of different stages of CHB patients in the test group

Twenty-one patients with the IT stage and 55 patients with the IC stage constituted the 76 HBeAg-positive patients. Significant differences in ALT levels, AST levels, HBV DNA, HBsAg, HBeAg, HBcAb, and platelet (PLT) counts were found between the two groups. There was no significant difference in age, sex, albumin, or total bilirubin between the two groups (Table 2). The semiquantitative levels of HBeAg are significantly greater in patients with the IT stage than in patients with the IC stage: 1588.93 [1433.06, 1799.06] S/CO and 740.55 [53.01, 1169.98] S/CO, respectively (p< 0.001). Univariate logistic analysis showed that HBV DNA, HBsAg, HBeAg, HBcAb, and PLT were correlated with the IT stages, but ALT levels were not correlated with the IT stage. The use of ALT levels as the reference variable resulted in its exclusion from significance testing. Multivariate logistic analysis revealed that HBeAg was independently correlated with disease stage, as shown in Table 3. Fourteen patients in the ICO stage and 28 patients in the IR stage constituted the 42 HBeAg-negative patients. Univariate analysis revealed a significant difference in HBV DNA and HBsAg between the ICO and IR stages, except for ALT levels. There was no significant difference in

Table 1 Baseline data for collected patients

Variable		Test group ($n = 118$)	Validation group($n = 73$)
Sex (Male/Female)‡		83/35	44/29
Age*		43 (11)	37 [31, 47]
ALT(U/L)#		53.50 [33.50, 75.25]	43 [22.15, 123.6]
AST(U/L)#		43.00 [29.00, 59.00]	29.80 [20.35, 61.40]
Albumin(g/L)*		41.65 (4.10)	41.5 (2.97)
Platelet ($\times 10^9$/L)#		174.00 [133.00, 207.00]	163.00 [131.50, 206.00]
Total bilirubin(μmol/L)#		14.10 [11.2, 18.95]	12.40 [8.35, 17.65]
HBVDNA(logIU/ml)#		7.06 [5.55, 7.94]	6.24 [3.01, 7.77]
HBsAg(logIU/ml)#		3.52 [2.98, 4.32]	3.40 [2.40, 4.45]
HBeAg(S/CO)#		81.63 [0.43, 1364.44]	88.12 [0.37, 1142.67]
HBcAb(S/CO)#		8.91 [7.53, 9.90]	9.18 [8.21, 10.3]
Inflammation grade‡	G0	0(0)	2(2.7)
	G1	36 (30.5)	32 (43.8)
	G2	79 (67)	22 (30.1)
	G3	3 (2.5)	12 (16.4)
	G4	0(0)	5(7.0)
Fibrosis stage‡	F0	1 (0.9)	15 (20.6)
	F1	81 (68.6)	32 (43.8)
	F2	35 (29.6)	21 (28.8)
	F3	1 (0.9)	2 (2.7)
	F4	0(0)	3(4.1)

Legend:

* Mean

± standard deviation

‡ Percentage

Median and interquartile range

age, sex, albumin, total bilirubin, PLT count, or HBcAb between the two groups (Table 4).

Comparative analysis between different stages of CHB patients in the validation group

Eleven patients in the CHB immune tolerance stage and 29 patients in the immune clearance stage were included in the validation group. The results of univariate analysis revealed significant differences in the levels of ALT levels, AST levels, HBV DNA, HBsAg, and HBcAb between the two groups of patients ($p < 0.05$). The level of HBeAg in the immune tolerance stage is higher than that in the immune clearance stage. However, the difference was borderline significant ($p = 0.087$). No difference in the PLT was found between the immune tolerance stage and the immune clearance stage ($p = 0.473$) (Table 5). Twenty-three patients in the immune control stage and 10 patients in the immune reactivation stage of CHB were included in the validation group. The results revealed significant differences in the levels of ALT levels, AST levels, HBV DNA and HBsAg between the two groups of patients ($p < 0.01$) (Table 6).

ROC curve analysis of the IT and IC stages of CHB patients in the test and validation groups

ROC curve analysis of the indicators of the IT and IC stages was performed via MedCalc software. The AUC was 0.806 (95% confidence interval (CI): 0.699–0.888) when HBV DNA was used as a variable in the test group. The cutoff value under the maximum Youden index was 7.509 log IU/ml for HBV DNA. The sensitivity and specificity were 56.36% and 100.00%, respectively (Fig. 1A). The cutoff value of the HBsAg concentration was more than 4.006 logIU/ml. The AUC was 0.912 (95% CI: 0.824–0.965). The sensitivity and specificity were 70.91% and 100.00%, respectively (Fig. 1B). The cutoff value of the HBeAg concentration was 1335.57 S/CO. The AUC was 0.921 (95% CI: 0.836–0.971). The sensitivity and specificity were 80.0% and 100%, respectively (Fig. 1C). HBcAb was used as a variable to differentiate the IT stage from the IC stage, yielding an AUC of 0.778 (95% CI: 0.665–0.868) with a maximum cutoff of 7.1 (S/CO) (Fig. 1D). The cutoff value of the PLT under the maximum Youden index was 161 $\times 10^9$/L. The AUC of the PLT was 0.720 (95%

Table 2 Comparative analysis of IT stage and IC stage of patients with HBeAg-positive CHB in test group

Variables		Overall($n=76$)	IT stage ($n=21$)	IC stage ($n=55$)	P
Sex(Male/Female)‡		49/27	11/10(52.4)	38/17	0.173
Age*		41.61(11.44)	38.67(7.38)	42.72(12.53)	0.168
ALT(U/L)#		57.00[36.25,75.00]	26.00[19.50, 32.00]	69.00[55.00, 93.00]	< 0.001
AST(U/L)#		43.00 [31.5, 59.75]	25.00 [22.5,28.00]	52.00 [41.00, 78.00]	< 0.001
Albumin(g/L)*		41.20 (4.28)	42.64(3.73)	40.65 (4.37)	0.069
Platelet ($\times 10^9$/L)#		181.50[145.00, 211.50]	205.00[177.00,238.00]	165.00[134.00,207.00]	0.003
Total bilirubin(μmol/L)#		13.9[10.75,18.78]	13.30 [10.65, 16.65]	15.10 [10.9, 18.90]	0.267
HBVDNA (logIU/ml)#		7.76[6.98,8.25]	8.28[7.83,8.51]	7.35[6.27,7.96]	< 0.001
HBsAg (logIU/ml)#		3.97[3.23,4.61]	4.79[4.50,4.91]	3.49[3.05,4.27]	< 0.001
HBeAg(S/CO)#		1061.96[104.13,1490.79]	1588.93[1433.06,1799.06]	740.55[53.01,1169.98]	< 0.001
HBcAb(S/CO)#		8.81 [7.12,9.57]	6.88 [4.83,8.88]	9.11[7.94,9.79]	< 0.001
Inflammation grade ‡	G1	22 (29)	21 (100)	1 (1.8)	< 0.001
	G2	52 (68.4)	0 (0.0)	52 (94.6)	
	G3	2(2.6)	0 (0.0)	2 (3.0)	
Fibrosis stage‡	F1	55 (72.4)	21 (100.0)	34 (61.8)	0.004
	F2	20 (26.3)	0 (0.0)	20 (36.4)	
	F3	1 (1.3)	0 (0.0)	1 (1.8)	

Legend:

* Mean

± Standard deviation

‡ Percentage

Median and interquartile range

Table 3 Univariate and multivariate analyses between IT and IC stage of CHB patients in test group

Variable	Univariable Logistic analysis		Multivariable Logistic analysis	
	P	HR (95%CI)	P	HR (95%CI)
ALT	0.971	/		
AST	0.000	1.284 (1.137–1.450)	0.006	1.186 (1.049–1.341)
Platelet	0.048	0.991 (0.982–1.000)		
HBVDNA	0.001	0.139 (0.043–0.446)		
HBsAg	0.000	0.022 (0.003–0.159)		
HBeAg	0.001	0.994 (0.991–0.998)	0.037	0.997 (0.994–1.000)
HBcAb	0.003	1.368 (1.109–1.689)		

CI: 0.605–0.817). The sensitivity and specificity were 49.09% and 100.00%, respectively (Fig. 1E). ROC curve analysis of the prediction value of the IT stage in the validation cohort revealed that the AUC of HBsAg was 0.875 (95% CI: 0.765–0.985, $p < 0.01$). The sensitivity and specificity were 82.8% and 90.9%, respectively (Fig. 2A). The AUC of HBeAg was 0.846 (95% CI: 0.726–0.967, $p < 0.01$). The sensitivity and specificity were 75.9% and 100%, respectively (Fig. 2B). The AUC of HBV DNA was 0.740 (95% CI: 0.580–0.899, $p < 0.05$). The sensitivity and specificity were 51.7% and 100%, respectively (Fig. 2C).

ROC curve analysis of CHB patients in the ICO and IR stages in the test and validation groups

Analysis of the ROC curve was performed via MedCalc software. HBV DNA was used as a variable to differentiate the ICO and IR stages. The cutoff value for HBV DNA according to the maximum Youden index was 3.279. The AUC was 1.000 (95% CI: 0.916–1.000). The sensitivity and specificity were 100.00% and 28.57%, respectively (Fig. 3A). The cutoff value for HBsAg according to the maximum Youden index was 2.934. The AUC was 0.967 (95% CI: 0.860–0.998). The sensitivity and specificity were 89.29% and 18.12%, respectively (Fig. 3B). ROC curve analysis of HBsAg and HBV DNA was performed for patients in the immune control stage in the validation group. The results revealed that the AUC for HBsAg was 0.948 (95% CI: 0.870–1.000, $p < 0.01$). The sensitivity and specificity were 80.0% and 100.0%, respectively (Fig. 4A). The AUC for HBV DNA was 1.000 (95% CI: 1.000- 1.000, $p < 0.01$). The sensitivity and specificity were 100.0% and 100.0%, respectively (Fig. 4B).

Table 4 Comparative analysis of ICO stage and IR stage of patients with CHB in test group

Variable		Overall($n=42$)	ICO($n=14$)	IR ($n=28$)	P
Sex(Male/Female)‡		34/8	12/2	22/6	0.890
Age*		44.24(8.84)	46.50(8.74)	43.11(8.82)	0.246
ALT(U/L)#		49.00 [24.75, 77.25]	20.00 [15.75, 25.25]	61.00 [48.5, 109.25]	< 0.001
AST(U/L)#		40.50 [24.75, 52.25]	23.00 [21.50, 26.25]	50.00 [39.25, 76.75]	< 0.001
Albumin(g/L)*		42.46 (3.67)	42.73 (3.70)	42.33 (3.71)	0.743
Platelet ($\times 10^9$/L)#		153.5 (50.41)	173.71(55.23)	143.39(45.52)	0.065
Total bilirubin(μmol/L)#		14.4 [11.8, 21.1]	12.95 [10.58, 17.63]	15.80 [11.80, 22.40]	0.196
HBVDNA (logIU/ml)#		4.84 [2.72, 6.61]	2.03 [1.30, 2.84]	5.84 [4.76, 6.83]	< 0.001
HBsAg (logIU/ml)#		3.04 [2.34, 3.54]	1.96 [0.63, 2.84]	3.31 [3.03, 3.56]	< 0.001
HBcAb(S/CO)#		9.35 [8.29, 10.49]	9.73 [8.29, 10.59]	9.15 [8.14, 10.34]	0.408
Inflammation grade ‡	G1	14 (33.3)	14 (100.0)	0 (0.0)	
	G2	27 (64.3)	0 (0.0)	27 (96.4)	
	G3	1 (2.4)	0 (0.0)	1 (3.6)	
Fibrosis stage‡	F0	1 (2.4)	1 (7.14)	0 (0.0)	
	F1	26 (61.9)	10 (71.43)	16 (57.1)	
	F2	15 (35.7)	3 (21.43)	12 (42.9)	

Legend:

* Mean

± standard deviation

‡ Percentage

Median and interquartile range

ROC curve analysis of a new prediction model for the IT stage in the test and validation groups

Multivariate analysis revealed that HBeAg was the only independent virological predictor. While HBsAg and HBV DNA were not independent predictors, they were included in the final model because of their well-established clinical relevance and because they are formally recognized as key phase-defining biomarkers in major clinical guidelines. Using three indicators (HBeAg, HBsAg, and HBV DNA), we established a new predictive model for the IT stage as follows: $Y = -0.896777 + (-0.634954)$*HBVDNA $+ 20.242533$*HBsAg $+ 3.063461$*HBeAg. The AUC was 0.923 (95% CI: 0.864–0.982, $p < 0.001$) when this prediction model was used in the test group (Fig. 5A). The sensitivity and specificity were 85.5% and 95.2%, respectively. The positive predictive value (PPV) of the model for IT was 91.3%. The AUC of the new model in the validation group was 0.890 (95% CI: 0.787–0.994, $p < 0.01$). The sensitivity and specificity were 79.3% and 100%, respectively (Fig. 5B). The PPV of the model for the IT is 85.7%.

Error rates of the new model for the prediction value of the IT stage in the test and validation groups

Twenty-one patients were classified into the immune tolerance stage according to liver histopathology, biochemical indicators and hepatitis B virus markers. According to the new model (NM), Guidelines of CMA (2019 version) (15), EASL (2017 version) (12) and AASLD (2018 version) (16), there were 23 patients, 28 patients, 33 patients and 43 patients, respectively, for which only biochemical indicators and hepatitis B virus markers are used to determine patients with the IT stage. The error rates were 4.65%, 11.62%, 23.26% and 46.51% when using the new model, CMA guidelines (2019 version), EASL guidelines (2017 version) and AASLD guidelines (2018 version), respectively (Fig. 6A). Different standards were used to distinguish patients with immune tolerance. The validation results revealed that 7 patients were in the IT stage according to the NM, 16 patients were in the IT stage according to the CMA guidelines (2019 version), 19 patients were in the IT stage according to the EASL guidelines (2017 version), and 20 patients were in the IT stage according

Table 5 Comparation analysis between immune tolerance and immune clearance stage in validation group

Variable		Overall($n=40$)	IT($n=11$)	IC ($n=29$)	p
Sex(Male/Female)‡		29/11	8/3	21/8	0.173
Age *		33 [30.00, 37.75]	34 [30, 41]	33 [29, 37]	0.168
ALT(U/L)#		73.80[34.35,269.17]	28.80[23.80, 33.50]	129.5[59.85, 307.4.00]	< 0.001
AST(U/L)#		41.35 [24.1, 108.52]	20.70 [19.6,26.20]	58.70 [38.55, 136.80]	0.006
Albumin(g/L)*		41.80 (2.93)	40.47(2.50)	42.31 (2.50)	0.076
Platelet ($\times 10^9$/L)#		171.00[146.75, 220.5]	203.00[160.00,237.00]	171.00[143.00,202.00]	0.473
Total bilirubin(μmol/L)#		12.25[8.02,16.1]	8.10 [5.4, 13.6]	13.5 [9.8, 19.15]	0.241
HBVDNA (llogIU/ml)#		7.73[7.12,8.00]	7.82[7.69,8.37]	7.34[6.28,7.94]	0.001
HBsAg (llogIU/ml)#		4.34[3.58,4.68]	4.75[4.61,4.98]	3.98[3.39,4.48]	< 0.001
HBeAg(S/CO)#		1029.36[248.67,1367.4]	1588.93[1433.06,1799.06]	735.87[133.34,1144.76]	0.087
HBcAb(S/CO)#		8.92 [7.46,10.05]	7.43 [5.69,8.55]	9.33[8.4,10.57]	0.022
Inflammation grade‡	G0	1 (2.5)	1 (9.1)		< 0.001
	G1	10 (25)	10 (90.9)		
	G2	17(42.5)		17(58.6)	
	G3	8 (20)		8(27.6)	0.001
	G4	4 (10)		4(13.8)	
Fibrosis stage‡	F0	5 (12.5)	2(18.2)	3(10.4)	
	F1	20(50)	9(81.8)	11(37.9)	
	F2	11(27.5)		11(37.9)	
	F3	2(5)		2(6.9)	
	F4	2(5)		2(6.9)	

Legend:

*Mean

± standard deviation

‡ Percentage

Median and interquartile range

to the AASLD guidelines (2018 version). The error rates were 20.0%, 25.0%, 40.0%, and 45.0%, respectively, when using the NM, CMA guidelines (2019 version), EASL guidelines (2017 version), and AASLD guidelines (2018 version) (Fig. 6B).

Discussion

At present, the indicators used for staging chronic HBV infection include ALT levels, HBV DNA, HBeAg, HBsAg, and liver pathological changes. However, owing to the invasiveness of liver biopsy, most related studies do not include the liver pathological status, which affects the accuracy of the clinical staging of CHB patients to a large extent [4, 15]. This study analyzed the indicators used in the clinical staging of chronic HBV-infected patients on the basis of the observed liver histopathological changes in patients with chronic HBV infection. The biochemical and virological indicators commonly used in patients are different in various stages of CHB. The high level of HBeAg semiquantification is especially important for defining the IT stage in the test and validation groups. A new model combining three indicators (HBV DNA, HBeAg, HBsAg) was established to differentiate the IT and IC stages of CHB. The sensitivity and specificity of this new model were high in the test and validation groups. Although liver hardness measurement (LSM) and HBV RNA show great potential, the completeness of LSM data was insufficient in our cohort, and HBV RNA testing was not routinely performed. The combination of additional noninvasive tests (e.g., LSM) and HBV RNA should be considered in view of the inconsistency in liver pathological change assessment, invasiveness, and sampling error between liver biopsy and routine HBV RNA testing in future studies.

The large gap in the definition of the IT and IC stages in the different guild lines has influenced the comparison of the results of different studies and the evaluation of the effect of anti-HBV therapy [4, 13]. In this study, there were significant differences in the levels of ALT levels, HBV DNA, HBsAg, HBeAg, PLT count, and HBcAb between the IT and IC stages. Considering that HBeAg is a marker of the initiation of the immune response in

Table 6 Comparative analysis of CHB patients with immune control and reactivation stages in validation group

Variable		Overall($n=33$)	ICO($n=23$)	IR ($n=10$)	p
Sex(Male/Female)‡		15/18 (54.5)	10/13 (56.5)	5/5 (50)	0.739
Age *		43.00 [38, 51.5]	43.00 [39, 52]	46.00 [30.5, 52]	0.42
ALT(U/L)#		21.90 [13.3, 56.15]	19.00 [12.4, 22.5]	73.70 [56.37, 137.55]	0.003
AST(U/L)#		21.30 [18.8, 37.45]	20.3 [18.2, 21.6]	65.25 [40.22, 139.02]	0.007
Albumin(g/L)*		41.2 (3.03)	41.25 (3.37)	41.09 (2.2)	0.89
Platelet ($\times 10^9$/L)#		151.5 (46.46)	161(48.2)	129.7(35.19)	0.075
Total bilirubin(μmol/L)#		13.1 [9.05, 18.95]	11.2 [8, 18.1]	17.05 [11.95, 19.9]	0.072
HBVDNA (LogIU/ml)#		3 [2.69, 4.31]	2.69 [2.42, 3.01]	5.82 [4.32, 6.39]	< 0.001
HBsAg (LogIU/ml)#		2.41 [1.83, 2.97]	2.25 [1.64, 2.58]	3.29 [2.88, 3.69]	< 0.001
HBcAb(s/co)#		9.65 [8.48, 10.69]	9.25 [8.35, 10.32]	10.12 [8.78, 11.53]	0.737
Inflammation grade ‡	G0	1(3)	1(4.3)	0 (0.0)	< 0.001
	G1	22(66.6)	22(95.7)	0 (0.0)	
	G2	5(15.2)	0 (0.0)	5(50)	
	G3	4(12.1)	0 (0.0)	4(40)	
	G4	1(3)	0 (0.0)	1(10)	
Fibrosis stage‡	F0	10(30.3)	10(43.5)	0 (0.0)	0.001
	F1	12(36.4)	9(39.1)	3(30)	
	F2	10(30.3)	4(17.4)	6(60)	
	F4	1(3)	0 (0.0)	1(10)	

Legend:

*Mean

± standard deviation

‡ Percentage

Median and interquartile range

patients with CHB, a decrease in HBeAg levels suggests that CHB patients have entered the IC stage [19]. The fact that patients are HBeAg positive (i.e., S/CO > 1) and not the difference in HBeAg levels as a differentiation criterion of the IT and IC stages might result in the problem of inaccurate staging. On the basis of the pathological changes in the liver, this study revealed that the cutoff value of the semiquantitative level of HBeAg that differentiated the IT and IC stages was 1335 S/CO. This cutoff value of HBeAg also has good sensitivity and specificity in the validation group. The IT stage differentiated on the basis of the HBeAg cutoff value of this study might be more in line with the definition of the IT stage and more helpful in terms of guiding the treatment of CHB. However, our proposed HBeAg threshold values are dependent on the specific assay (Abbott Alinity) and patient population from China. These cutoffs need prospective clinical validation and possibly recalibration for different assay kits or cohorts.

The standards of HBV DNA established by the various guidelines are also inconsistent, ranging from 6 log IU/mL to >7.3 log IU/mL. The standard identified by the World Gastroenterology Organization (WGO) is more than 9 log IU/mL [20]. HBsAg levels at the IT stage are mostly greater than 4 log IU/mL. These relevant criteria lack evidence-based medical evidence of a large sample and whether it reflects the "immune tolerance" state. Hui et al. reported that patients with more than F1 liver fibrosis were excluded from the IT stage. Patients who remained in the IT stage presented with only mild lesions during the follow-up stage. The median serum HBV DNA concentration is 9.81 log copies/mL in 48 immune-tolerant patients [21]. The results of our study show that the cutoff value of HBV DNA is >7.51 log IU/mL, which is similar to the results reported by Hui et al. [21]. The EASL guidelines suggest that the level of HBsAg quantification should be high, but the specific values are not supported. Tseng et al. suggested that the level of HBsAg should be more than 5 log IU/mL [22]. This study revealed that the cutoff value for HBsAg quantification in patients with the IT stage is >4.01 log IU/mL. The AUC values for HBsAg quantification were 91.2% and 87.5% in patients in the test and validation groups, respectively. These results suggest that sufficiently high HBV DNA and HBsAg levels could also be indicators for distinguishing patients with the IT stage.

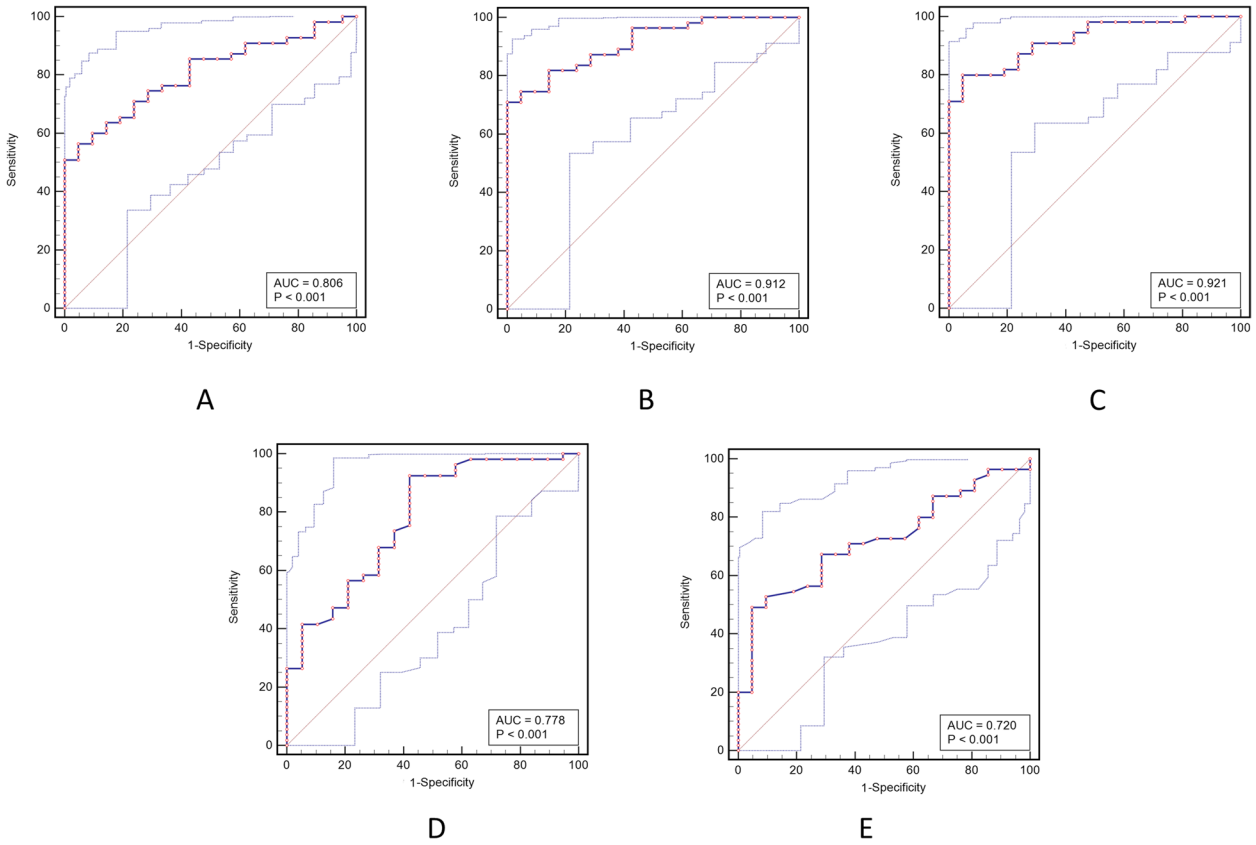

Fig. 1 ROC curve analysis of the IT and IC stages of CHB patients in the test group. Legend: A, ROC curve analysis of HBV DNA; B, ROC curve analysis of HBsAg; C, ROC curve analysis of HBeAg; D, ROC curve analysis of HBcAb; E, ROC curve analysis of the PLT count. IT: immune tolerance; IC: immune clearance

Although many studies have investigated alterations in immune function in chronic hepatitis B patients, no immune indicators for evaluating the immune tolerance stage have been used in the clinic [5, 6]. Moreover, there is a lack of accurate clinical models for evaluating immune tolerance stages. A predictive model for the immune tolerance stage was established by combining three indicators via single indicator analysis in this study. The AUC values were 92.3% and 89.0% in patients in the test and validation groups, respectively. Compared with the CMA standards (2019 version), EASL standards (2017 version), and AASLD standards (2018 version), the new prediction model had a significantly lower error rate. It is speculated that patients in the immune tolerance stage with persistently high levels of HBeAg, HBV DNA, and HBsAg but normal ALT levels have an extremely low risk of progressing to cirrhosis and HCC. However, once they transition to the immune clearance phase, the risk greatly increases, and timely antiviral treatment is needed. Currently, a follow-up study is being conducted for patients in the immune tolerance stage, with the expectation that future findings will provide better guidance for the clinical management of chronic HBV infection.

Studies have shown that the PLT gradually decreases in patients with liver fibrosis and is negatively correlated with the degree of liver fibrosis. The decline in the PLT was more obvious, especially in the stage of cirrhosis [23]. Anti-inflammatory agents, such as aspirin, have been shown to play a role in both animal models and clinical studies [24]. PLT count was independently associated with the IT and IC stages in chronic HBV-infected patients in our study. The PLT count of patients in the CHB IC stage was significantly lower than that of patients in the IT stage. These results suggest that the PLT count can be used as an indicator to differentiate between patients in the IT and IC stages. The reason may be related to the presence of obvious liver fibrosis in patients in the IC stage, and the PLT count may play an important role in the development of liver fibrosis and clinical evaluation. HBcAb is the earliest antibody produced after patients are infected with HBV; however, it is not a protective antibody [25]. Jia et al. [26] reported that the levels of HBcAb significantly varied at different

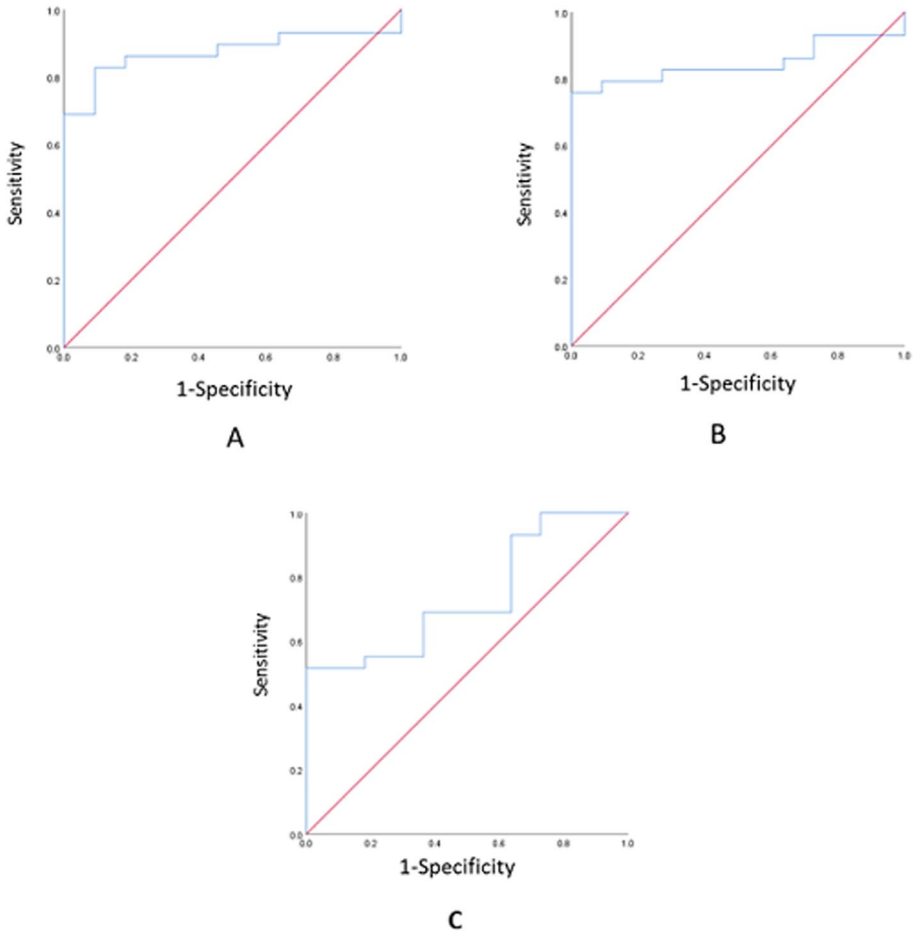

Fig. 2 ROC curve analysis of the IT and IC stages of CHB patients in the validation group. Legend: A, ROC curve analysis of HBsAg; B, ROC curve analysis of HBeAg; C, ROC curve analysis of HBV DNA. IT: immune tolerance; IC: immune clearance

stages in chronic HBV patients. This study revealed that the semiquantitative level of HBcAb in patients with the IT stage was lower than that in patients with the IC stage, which is similar to the results of Jia et al. No significant difference was found between the ICO and IR stages, which is inconsistent with the results of Jia et al. The reasons for the inconsistency might be related to the detection methods and sample differences. However, further studies are needed.

The levels of HBsAg and HBV DNA differ according to the current criteria for differentiating between patients in the ICO and IR stages [12, 16]. Some studies reported that the ICO statuses of CHB patients and HBeAg-negative CHB patients were differentiated but not better when HBsAg was lower than 3 log IU/mL and when HBV DNA was less than 3.3 log IU/mL [27]. However, liver pathological changes in their study were not included. Most current guidelines use this criterion to differentiate ICOs from IR patients [15–17]. Recent studies have revealed that patients with HBV DNA levels less than 3.3 log IU/mL still have a high incidence of cirrhosis and HCC, and these patients may also need therapy [28]. The results of this study show that the HBV DNA and HBsAg levels are significantly different between the ICO and IR stages and that there is a significant difference in liver pathological changes between the two groups of patients in the test and validation groups. Persistent negative HBV DNA and loss of HBsAg are considered criteria for a clinical cure. The inconsistency between hepatitis B virus clearance and pathological changes in the liver, considering the mechanisms of HBV clearance, may be cytotoxic or noncytotoxic [29]. Therefore, the assessment of liver pathological changes may not be suitable for distinguishing between the ICO stage and the IR stage and needs to be combined with clinical indicators for a comprehensive evaluation. Cutoffs of HBsAg < 3 log IU/ml and HBV DNA < 1.3 log IU/mL are recommended for patients in the ICO stage according to the CMA guidelines (version 2022) [30]. However, many patients

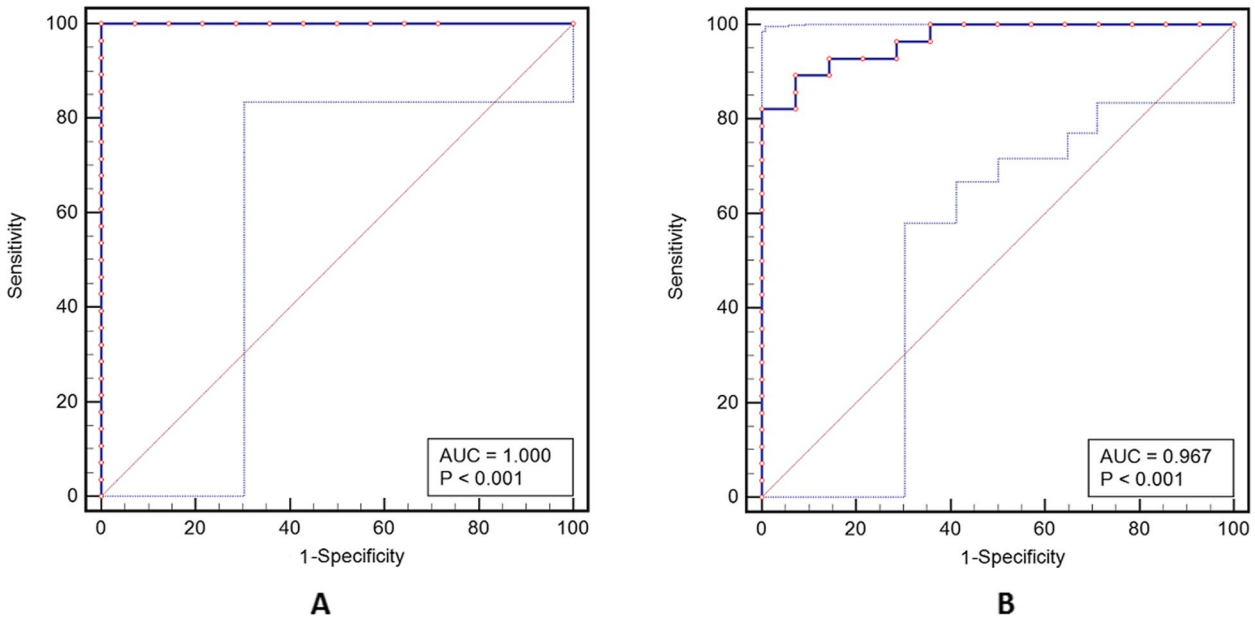

Fig. 3 ROC curve analysis of the ICO and IR stages of CHB patients in the test group. Legend: A, ROC curve analysis of HBV DNA levels; B, ROC curve analysis of HBsAg in the test group. ICO: immune control; IR: immune reactivity

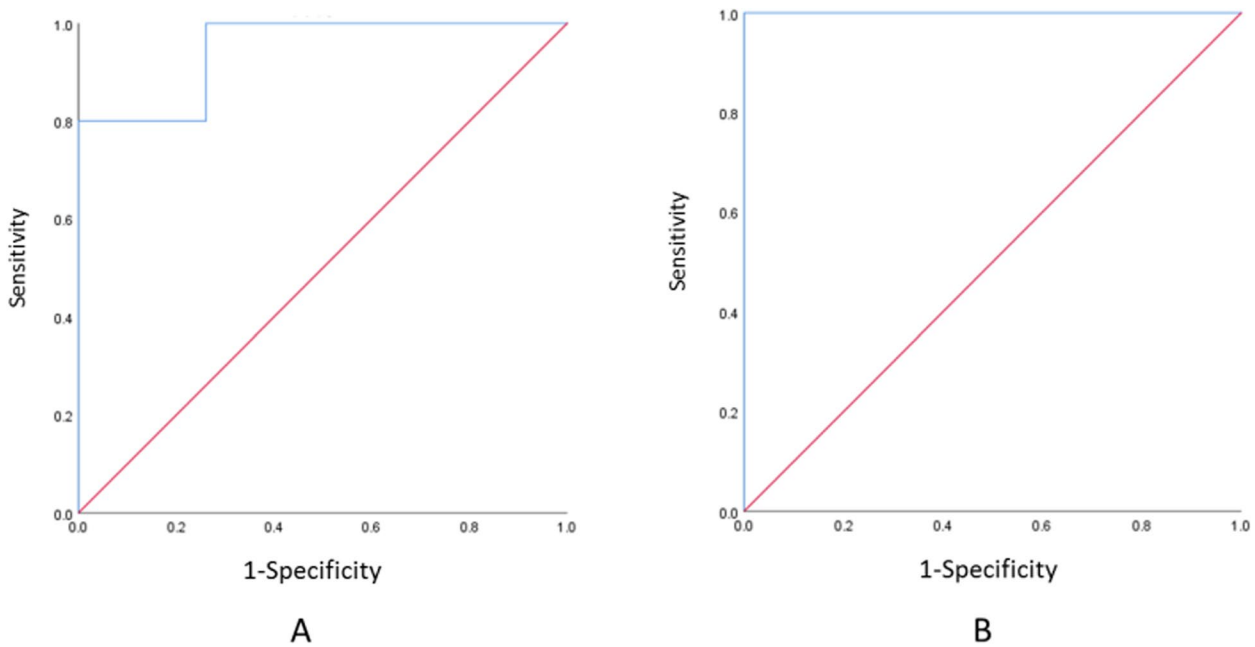

Fig. 4 ROC curve analysis of the ICO and IR stages of CHB patients in the validation group. Legend: A, ROC curve analysis of HBV DNA for validation; B, ROC curve analysis of HBsAg in the validation group. ICO: immune control; IR: immune reactivity

still have high levels of HBsAg (> 3 log IU/ml) after negative HBV DNA results due to the influence of HBV DNA integration.

On the basis of the aforementioned findings, we recently proposed a revised staging system for the natural history of chronic hepatitis B aimed at guiding antiviral therapy decisions [31]. However, this study has several limitations. First, this study was a retrospective analysis from two centers in China. The small size, older age and use of only samples from China make it difficult to

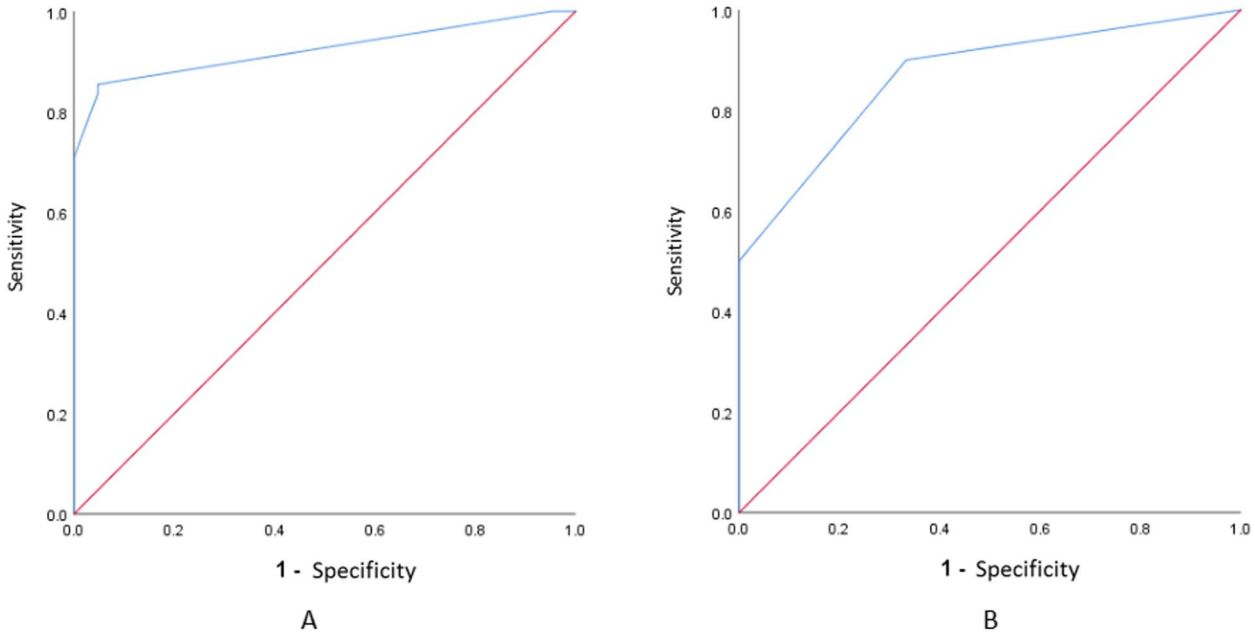

Fig. 5 ROC curve analysis of the new model for the prediction of the IT stage in the test and validation groups. Legend: A, ROC curve analysis of the new prediction model for the IT stage in the test group; B, ROC curve analysis of the new predictive model in the validation group

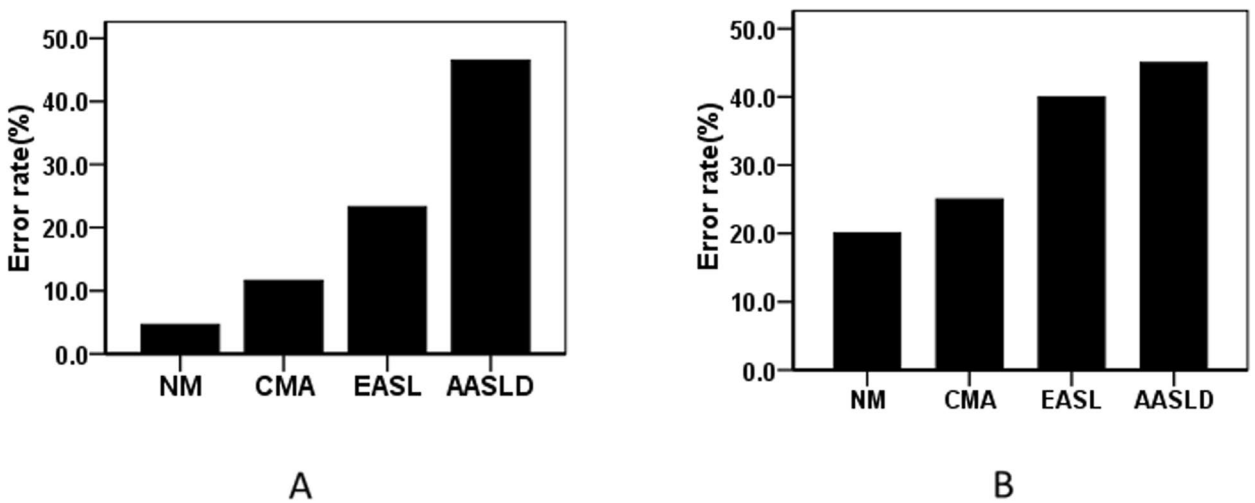

Fig. 6 Error rates between the new model and other guidelines in the test and validation groups. Legend: A: Error rates by different criteria in the test group; B: Error rates by different criteria in the validation group. NM: new model; CMA: Chinese Medical Association; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases

generalize these findings to all patients. Therefore, further sample expansion and multicenter study validation are needed. Second, some samples presented mild fatty changes, which may have affected the accuracy of this study. The implementation of our model would require laboratories to have quantitative HBsAg and semiquantitative HBeAg assays available. The detection methods for HBeAg used in this study are semiquantitative, which requires validation using unified quantitative detection methods in the future. Finally, a dynamic observational analysis of patient outcomes was not performed in this study. Further follow-up observations could provide insights into the utility of the new predictive model in clinical practice, especially in relation to patient outcomes such as the development of cirrhosis or HCC, in future studies.

In conclusion, this study optimized the levels of HBsAg, HBV DNA, and HBeAg on the basis of liver pathological changes and proposed new cutoff values for the staging of chronic HBV infection, especially when the HBeAg semiquantitative value is greater than 1335 S/CO, which can help to clinically differentiate patients with the true IT stage. A predictive model for the immune tolerance stage was first established by combining three indicators. Compared with the other standards, the new prediction model has a significantly lower error rate. These results may provide more accurate criteria for the clinical staging and evaluation of the treatment response of patients with CHB.

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Authors' contributions
X.T. wrote the main manuscript text. L. W. and Z.B. prepared Figs. 1, 2, 3, 4, 5 and 6. H.R. AND W.J. collected the data. W.Q. and F.J.prepared the data. All authors reviewed the manuscript.

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Data availability
All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethical Commission of Taizhou Hospital of Zhejiang Province(ethical approval number: 2023–03-47–01) and Ethics Committee of Nanjing Drum Tower Hospital, Jiangsu Province(ethical approval number: 2008022). All patients signed an informed consent form. This study adhered to the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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